ATTORNEY DOCKET No.: 2003946-0056 (ANDI-001-C1US)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Chiba, *et al.*	Examiner:	Robert J. Balls
Serial No.:	10/657,910	Art Unit:	1625
Filing Date:	September 9, 2003		
Title:	MACROCYCLIC COMPOUNDS USEFUL AS PHARMACEUTICALS		

Mail Stop: Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Dr. John (Yuan) Wang, declare as follows:

1. I am a co-inventor of the subject matter disclosed and claimed in United States patent application, Serial Number 10/657,910, filed September 9, 2003, and entitled "MACROCYCLIC COMPOUNDS USEFUL AS PHARMACEUTICALS".

2. I received a doctorate in organic chemistry from Harvard University in 1990. My curriculum vitae is attached as Exhibit 1.

3. I have extensive expertise in macrolide chemistry and I am a co-author of a number of articles relating to that field. A list of some of these is included in my CV.

4. I am Senior Scientist at Eisai Research Institute, a wholly owned subsidiary of the Assignee of the above-referenced patent application. I was the Leader of the Medicinal Chemistry Team that invented and developed the compounds of the subject application. As such, I was responsible, in part, for designing compounds of the invention, devising synthetic approaches for making these compounds, and coordinating synthetic research efforts to prepare

compounds of the invention for biological testing. In addition, I worked in close collaboration with the Biology Research Team that investigated the biological properties of the compounds of the subject application. Therefore, I am well-versed with the chemical and biological properties of compounds of the subject application, as well as the various assays that were used to assess the compounds biological properties.

5. I have read the Office Action mailed March 16, 2006, and understand that the Examiner has rejected the claims. In particular, I understand that the Examiner has taken the position that the claimed compounds are obvious in light of references by Dombrowski and Patani. One purpose of my Declaration is therefore to establish that the claimed compounds, for which R_2 = methyl, have distinct and unexpected properties as compared with any compounds discussed by Dombrowski or Patani.

6. I further understand that the Examiner has questioned whether inventive compounds for which R_9 is other than hydroxy have biological activity. Another purpose of this Declaration, therefore, is to confirm that such compounds do have NF-κB inhibitory activity, as described in the patent application.

7. NF-κB is a critical nuclear factor that regulates various genes involved in immune and inflammatory responses (see, for example, Ghosh *et al.*, *Annu Rev Immunol.* **1998,** 16, 225). It is well believed that TNFα gene transcription is regulated by NF-κB activation (see, for example, Drouet *et al.*, *J. Immunol.* **1991,** *147,* 1694). The inhibitory effect of test compounds on NF-κB activation was assessed by evaluating their effect on expression of a TNFα-PLAP (placental alkaline phosphatase) reporter. NF-κB is not believed to regulate expression from the β-actin promoter. Thus, the specificity of any inhibitory effect of test compounds observed with the TNFα-PLAP construct was evaluated by also assessing their effect on expression of a β-actin - PLAP reporter. All the compounds that were prepared within the scope of the present claims (many of which are depicted on pages 71-90 of the application) were evaluated in the TNF-α PLAP and β-actin PLAP systems.

Specifically, the effect of test compounds on TNF-α and β-actin PLAP transcription was measured in THP-1-33 and B164 cells, respectively, as follows:

A TNFα-PLAP plasmid (TNFα-promoter + 5'-UTR (1.4 kb) + PLAP + SV40 polyA + PGK-neo, Goto *et al., Mol. Pharmacol.* **1996,** *49*, 860-873) was constructed with slight modification in which TNFα-3'-UTR (772 b.p.) was inserted between PLAP and SV40 polyA (TNFα-promoter + 5'-UTR (1.4 kb) + PLAP + TNFα-3'-UTR + SV40 polyA + PGKneo). The THP-1-33 cells were then established by stably transfecting the modified TNFα-PLAP plasmid into THP-1 cells (human acute monocytic leukemia). In order to simultaneously evaluate non-specific effects of test compounds on transcription, B164 cells were also established by stably transfecting β-actin-PLAP plasmid (β-actin-promoter (4.3 kb) + PLAP + SV40 polyA + PGKneo) into THP-1 cells. THP-1-33 cells (TNFα-PLAP) produce PLAP activity by stimulation with LPS. On the other hand, B164 cells (β-actin-PLAP) constantly produce PLAP activity without stimuli.

THP-1-33 cells and B164 cells were maintained in RPMI1640 containing 10% heat-inactivated endotoxin-free fetal bovine serum (FBS) and G418 (1 mg/ml). These cells were seeded at a density of 1.0×10^4 cells/well onto 96-well plate, then were cultured in the presence or absence of test compounds for 30 min, followed by stimulation with 100 ng/ml of lipopolysaccharide (*E.coli* 0127:B08 or 011:B4). After the cultivation for 40-48 hrs, culture supernatant was harvested and alkaline phosphatase acivity in the supernatant was measured.

Alkaline phosphatase activity was quantified with the use of a chemiluminescent substrate, 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2'-adamantane]. To inactivate tissue-nonspecific alkaline phosphatase mainly derived from FBS, samples were heated at 65°C for 30 min before the chemiluminescent assay. Aliquots of 10 μl of culture supernatant were mixed with 50 μl of assay buffer (0.28 M Na_2CO_3-$NaHCO_3$, pH 10.0, containing 8 mM $MgSO_4$) in a 96-well Microlite™ plate (opaque), and then 50 μl of chemiluminescent substrate was added and mixed. After 60 min incuvation at room temperature, steady state chemiluminescence was measured with a microplate luminometer.

The PLAP activity of each sample was calculated as follows:

TNFα-PLAP	% of control = (A-B) x 100/ (C-B)
β-actin-PLAP	% of control = (A) x 100/ (C)

A: sample/chemiluminescence of the sample cultured with the test compound & stimulated with LPS

B: blank/chemiluminescence of unstimulated sample

C: control/chemiluminescence of the sample cultured with LPS

The IC_{50} value of each test compound was calculated from dose-inhibitory response curve.

Most test compounds were found to exhibit NF-κB inhibitory activity. A sample of the data that were collected is shown as Exhibit 2. As can be seen, many compounds in which R_9 is other than hydroxy have activity.

Copies of the references cited in this section are provided as Exhibit 5.

8. A number of compounds were also tested *in vivo* in mice, using a skin inflammation model to quantify the topical effectiveness of a compound. The data that was generated is expressed as % inhibition in swelling and % inhibition in MPO, and is shown as Exhibit 3.

The experiments were conducted as follows:

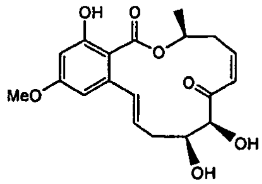
% inhibition in swelling

Six hours after croton oil application, ear thickness was measured. The intensity of the elicited inflammatory swelling was calculated as the difference in the thickness between the croton oil-treated (right) and the untreated (left) ear of the same mouse. The % inhibition in swelling was calculated (the higher the number, the more effective the compound).

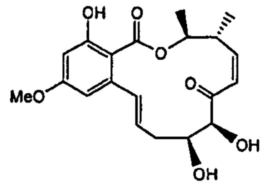
% inhibition in MPO

MPO (Myeloid peroxidase) is an enzyme and MPO activity as an indicator of neutrophil infiltration (mechanism of inflammatory response). The ears were excised and subsequently the ear skin homogenate was prepared for the evaluation of MPO activity. The % inhibition in MPO was calculated (the higher the number, the more effective the compound).

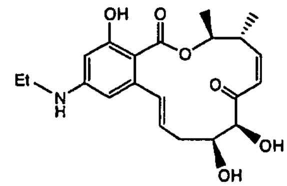
9. The stability of two compounds of the invention, ER-803064 and ER-806201, was compared with that of a prior art compound, F-152. In particular, the plasma concentration of each compound was monitored in a mouse. The compound structures are shown below.



F-152



ER-803064



ER-806201

The experiment was conducted as follows:

Both the heparinized mouse blood (5 mL), obtained by cardiovascular punch, and PBS were spiked with the methanol stock solution of F-152 (10 mg/mL) at a ratio of 1/100 (v/v). The spiked blood and PBS were incubated in a rocking waterbath at 37°C, and sampled at various time points (0-time, 15 min, 30 min, 1 h, 2 h, and 16 h). Each sample (250 µL) was mixed with 500 µL of methanol. The mixtures were centrifuged following vortex. The resulting supernatants, after being filtered through a syringe filter (0.25 µm), were analyzed using a HPLC-UV system with a reverse phase setting, i.e. Waters C18 column and gradient mobile phases. F-152 was monitored at the UV absorbance of 275 nm with the respective LC retention time.

The pharmacokinetic (PK) profiles (plasma concentration vs time) are provided as Graphs 1-3 in Exhibit 4.

The plasma concentration of F-152 over time is set out in Graph 1. As shown in Graph 1, the plasma concentration in mouse (I.V. administration) of F-152 noticeably drops within an hour. This compound does not have a C4 (position R_2) methyl. In contrast, the data for two compounds with a methyl at C4 (position R_2) is qualitatively different.

The plasma concentration of ER-803064 over time is set out in Graph 2. As shown in Graph 2, a much longer detectable plasma level was observed for ER-803064, which has a methyl at C4 (position R_2), as compared to F-152, which lacks a methyl group at position R_2.

Graph 3 indicates that the metabolic stability improvement was also maintained for ER-806201. This compound also has a methyl at C4 (position R_2) and has similar mouse PK data to ER-803064.

The data shows that F-152 is metabolically unstable (it is believed that the C5-C6 enone isomerizes quickly in mouse plasma). The data also demonstrates that the presence of a methyl at C4 (position R_2) imparts significantly higher plasma stability to compounds of the invention.

10. I, John (Yuan) Wang, declare that all statements made herein of my own knowledge are true and that these statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like are made punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patents that may issue thereon.

John Wang

John (Yuan) Wang, Ph.D.

8/16/06

Date

- EXHIBIT 1 -

John(Yuan) Wang Ph.D.
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yuan_wang@eisai.com

Education:

6/90 Ph. D. in Organic Chemistry, Harvard University

7/82 B.S. in Chemistry, Fudan University, P. R. China

Career Experience:

5/03-Present Chemistry Leader for various oncology projects.

4/97-5/03 Senior Scientist at Eisai, Chemistry leader of NF-κB inhibitor project.

9/96-4/97 Scientist at Eisai, Chemistry Project leader of Endotoxin Antagonist project.

6/94-9/96 Associate Scientist at Eisai, Research topic for last two years: synthesis of the anticancer natural product, right-half Halichondrin B and its analogs.

6/90-6/94 Senior Research Chemist at Eisai, Research Topic: Synthesis of Lipid A Antagonist E5531 (entered clinic Phase II), and related lipid A analogs.

1/86-6/90 Research Assistant under Prof. Yoshito Kishi. Thesis Topic: Synthetic And Conformational Studies of *C*-1,4-Disaccharides

1/84-1/86 Research Assistant under Prof. David Evans, Research Topic: Asymmetric intramolecular Diels-Alder Chemistry.

Personal Data:

US citizen

References: Available on request

Professional affiliations:

Member of American Chemical Society since 1986
Member of American Association of Cancer Research since 2005

Publications:

1) MEK/multi-kinase inhibitor for cancer: patent under preparation

2) Patent: U.S.S.N. 10/507,607(Sept. 8, 2004, international filing date March 7, 2003), U.S.S.N. 10/657,910 (Sept. 9, 2004)

3) Structually Simplified Macrolactone analogs of Halichondrin B B.M. Seletsky, **Y. Wang**, Lynn Hawkins, M. H. Palme, G.J. Habgood, L.V. DiPietro, M.J. Towle, K. A. Salvato, B. F. Wels, K. K. Aalfs, Y. Kishi, B.A. Littlefield and M.J. Yu Bioorganic & Medicinal Chemistry Letters **14**, 5547-5550 (2004)

4) Structure-Activity Relationship of Halichondrin B Analogues: Modifications at C.30-C.38 **Yuan Wang**, Greg J. Habgood, William J. Christ, Bruce A. Littlefield and Melvin J. Yu Bioorg. & Med. Chem. Lett. **10** (10) 1029-1032 (2000)

5) *In vitro* and *in vivo* Anticancer Activities of Synthetic Macrocyclic Ketone Analogs of Halichondrin B Murray J. Towle, Kathleen A. Salvato, Jacqueline Budrow, Bruce F. Wels, Galina Kuznetsov, Kimberley K. Aalfs, Susan Welsh, Wanjun Zheng, Boris M. Seletsky, Monica H. Palme, Gregory J. Habgood, Lori A. Singer, Lucian V. DiPietro, **Yuan Wang**, Jack J. Chen, David A. Quincy, Ashley Davis, Kentaro Yoshimatsu, Yoshito Kishi, Melvin J. Yu and Bruce A. Littlefield[2] Cancer Res.**61**, 1013-1021, 2001

6) A Practical Modification to the Kishi Synthesis of the C27-C38 Fragment of Halichondrin B **Yuan Wang***, William Christ, Greg Habgood, Andrea Robidoux, Melvin Yu. Poster Presentation (abstract 200, Division of Organic Chemistry) at ACS Spring National meeting 2000 at San Francisco

7) E5531, a Pure Endotoxin Antagonist of High Potency W. Christ, O. Asano, A. L. C. Robidoux, M. Perez, **Y. Wang**, G. R. Dubuc, W. E. Gavin, L. D. Hawkins, P. D. McGuinness, M. A. Mullarkey, M. D. Lewis, Y. Kishi, T. Kawata, J. R. Bristol, J. R. Rose, D. P. Rossignol, S. Kobayashi, I. Hishinuma, A. Kimura, N. Asakawa, K. Katayama, and I. Yamatsu ***Science***, 1995, **268,** 80-83.

8) Total Synthesis of the Proposed Structure of *Rhodobacter sphaeroides* Lipid A Resulting in the Synthesis of New Potent Lipopolysaccharide Antagonists W. Christ, P. McGuiness, O. Asano, **Y. Wang**, M. Mullarkey, M. Perez, L. Hawkins, G. Dubuc, and A. Robidoux. ***J. Am. Chem. Soc***. 1994, **116**, 3637.

9) Preferred Conformation of *C*-Glycosides. 8. Synthesis of 1,4-Linked Carbon Disaccharides **Y. Wang**, S. A. Babirad, and Y. Kishi. ***J. Org. Chem***. 1992 **57**, 468.

10) Preferred Conformation of *C*-Glycosides. 9. Conformational Analysis of 1,4-Linked Carbon Disaccharides **Y. Wang**, P. G. Goekjian, D. M. Ryckman, W. H. Miller, S. A. Babirad, and Y. Kishi. ***J. Org. Chem.*** 1992 **57**, 482.

11) Preferred Conformation of *C*-Glycosides. 5. Experimental Support for the Conformational Similarity between C- and O-Disaccharides W. H. Miller, D. M. Ryckman, P.G. Goekjian, **Y. Wang**, and Y. Kishi. ***J. Org. Chem.*** 1988 **53**, 5580.

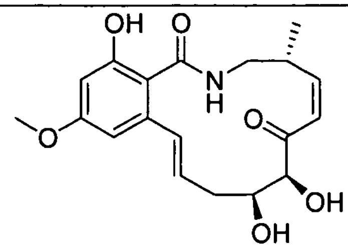
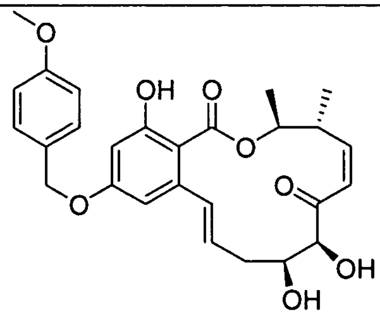
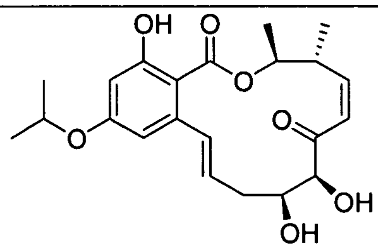
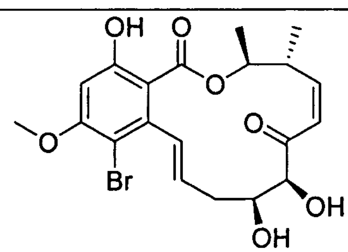
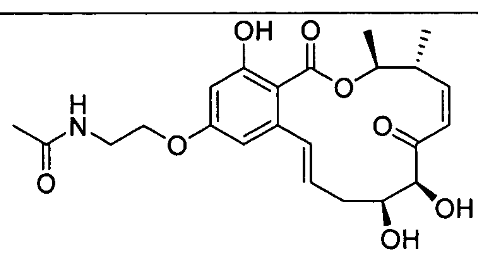
12) Preferred Conformation of *C*-Glycosides. 4. Importance of 1,3-diaxial-like Interactions around the Nonglycosidic Bond: Prediction and Experimental Proof **Y. Wang**, P. G. Goekjian, D. M. Ryckmann, and Y. Kishi ***J. Org. Chem.*** 1988 **53**, 4151

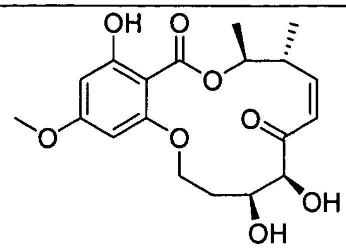
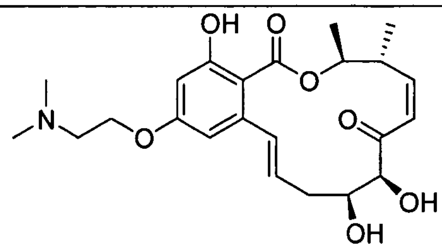
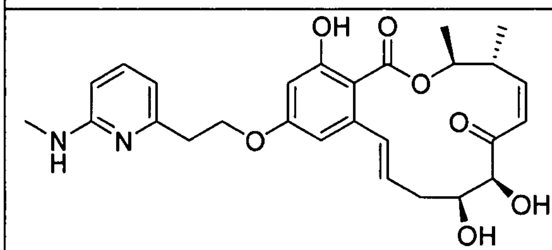
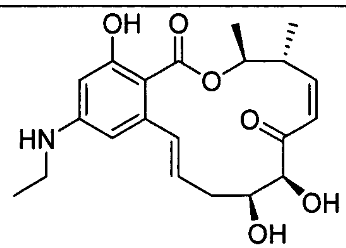
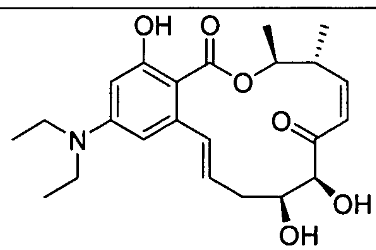
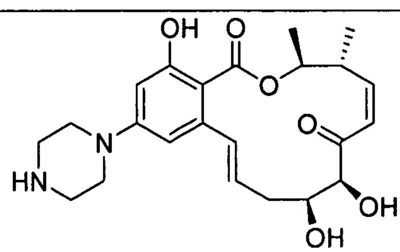
13) Preferred Conformation of *C*-Glycosides. 3. Preferred Conformation of Carbon Analogues of 1,4-Disaccharides S. A. Babirad, **Y. Wang**, P. G. Goekjian, and Y. Kishi ***J. Org. Chem.*** 1987 **52**, 4825

14) Synthesis of *C*-Disaccharides S. A. Babirad, **Y. Wang**, and Y. Kishi ***J. Org. Chem.*** 1987 **52**, 1370

- EXHIBIT 2 -

Demonstration of the NF-κB inhibitory activity of compounds of the claimed invention

Compound Structure	Compound number	THP-1-33 cells	B164 cells
		TNFα-PLAP (nM)	β-Actin PLAP (nM)
	ER-804022	259 (n=3)	>10 000
	ER-804104	168 (n=3)	>10 000
	ER-804189	992 (n=2)	>10 000
	ER-804505	804 (n=5)	>10 000
	ER-804555	58 (n=19)	>10 000

	ER-804622	1 683 (n=1)	>10 000
	ER-804759	597 (n=6)	>10 000
	ER-805233	441 (n=1)	>10 000
	ER-806201	50 (n=17)	>10 000
	ER-806203	2 066 (n=5)	>10 000
	ER-806776	351 (n=1)	>10 000

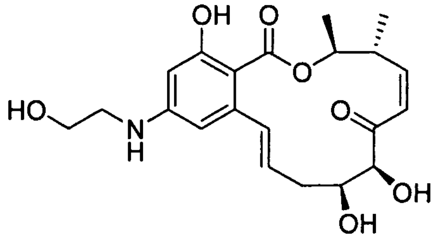
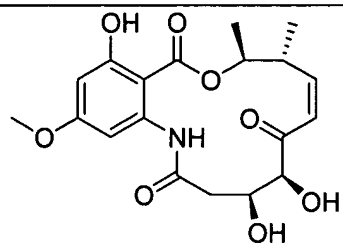
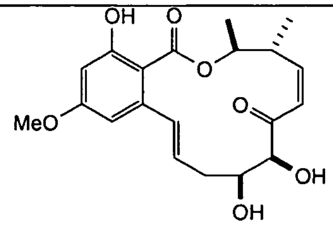
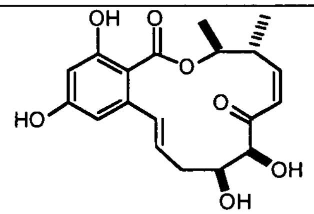
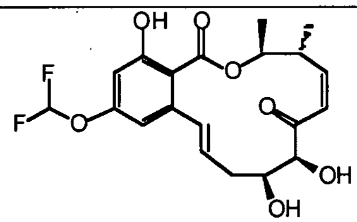
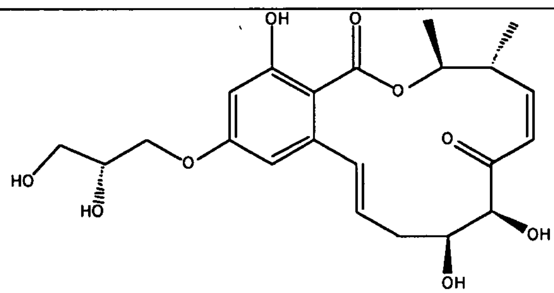
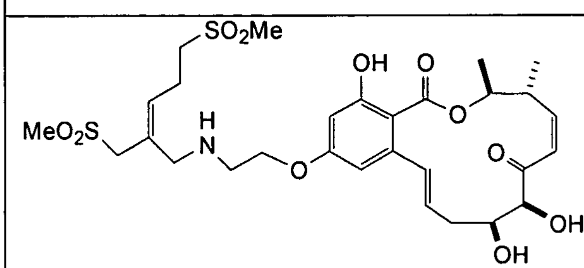
	ER-806907	40 (n=4)	>10 000
	NF2306	293 (n=8)	>10 000

Exhibit 2

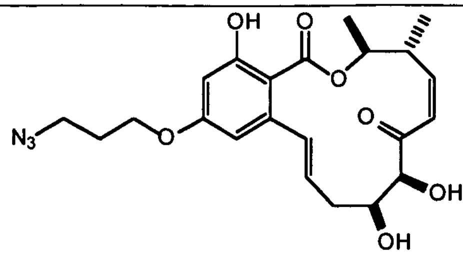
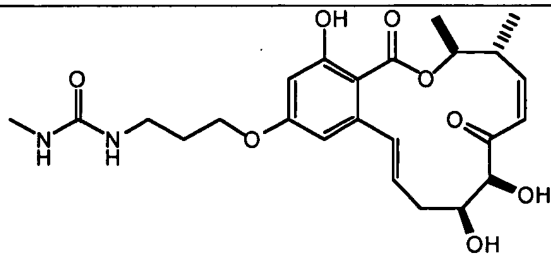
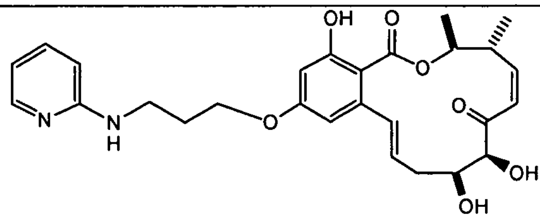
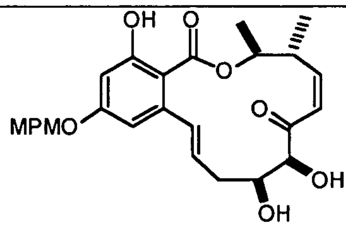
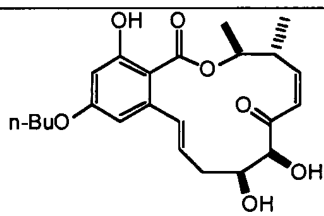
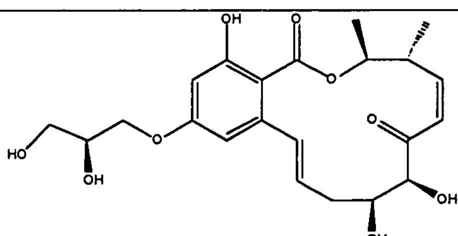
4092373v1

- EXHIBIT 3 -

Demonstration of the *in vivo* activity of compounds of the claimed invention

Compound Structure	Compound number	Topical effectiveness @ 0.3%	
		I % in swelling	I % in MPO
	ER-803064	31	31
	ER-804131	23	38
	ER-804446	15	32
	ER-805229	28	12
	ER-805218	24	10

4092373v1

	ER-804734	6	23
	ER-804779	21	4
	ER-805190	15	13
	ER-804104	8	12
	ER-804504	17	2
	ER-805232	14	0

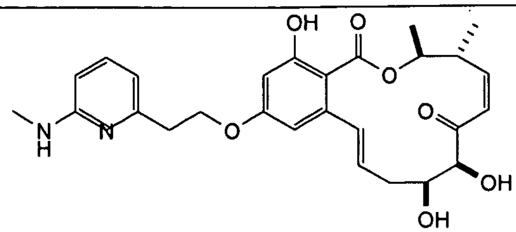
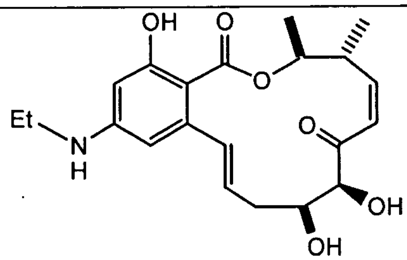
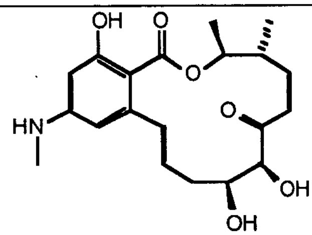
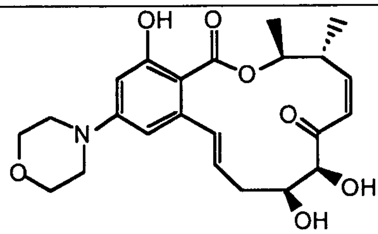
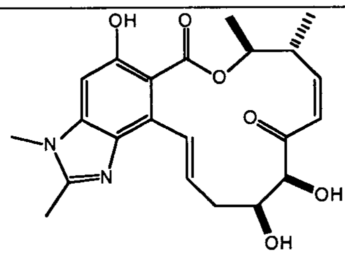
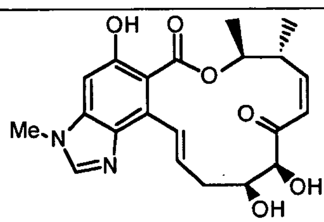
	ER-805233	0	0
	ER-806201	73	67
	ER-805940	67 (average n=3)	54 (average n=3)
	ER-806621	80	36
	ER-805911	47 (average n=2)	46 (average n=2)
	NF2561	51	29

Exhibit 3

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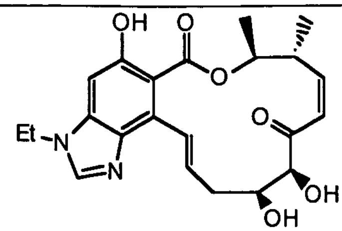
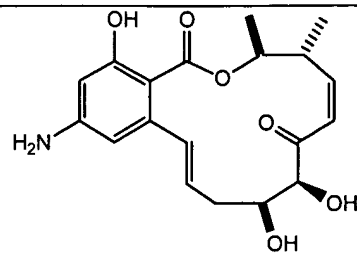
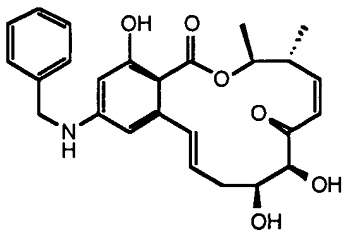
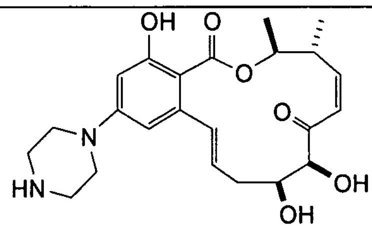
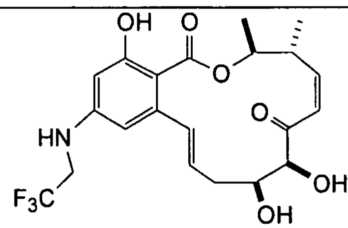
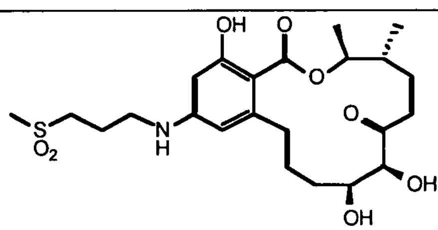
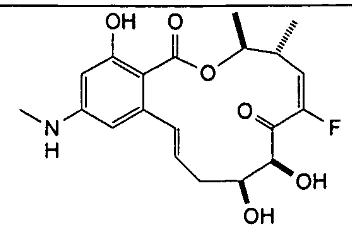
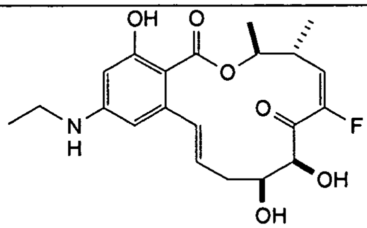
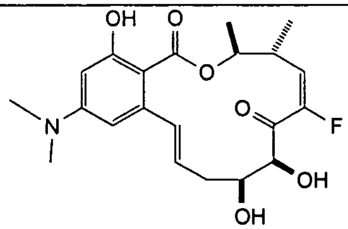
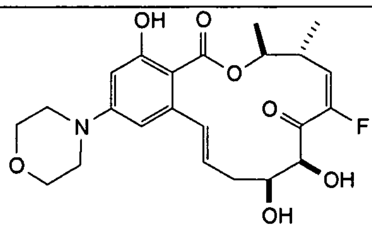
	ER-805977	47	32
	ER-804758	32	28
	ER-806624	18	17
	ER-806776	37	4
	ER-806795	11	0
	ER-805998	9	0

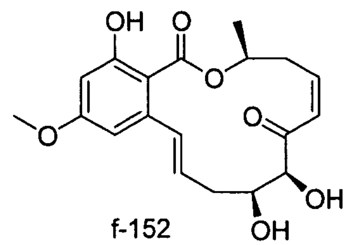
	ER-806821	62	67
	ER-807563	60	51
	ER-808064	43 (average n=2)	46 (average n=2)
	ER-807551	34	34

- EXHIBIT 4 -

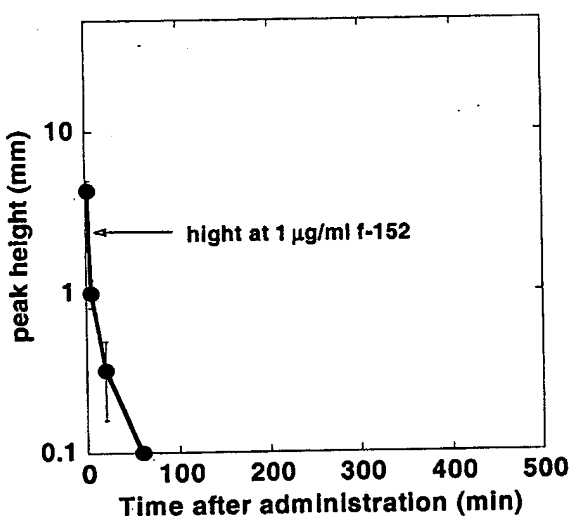
Demonstration of the improved stability of compounds of the claimed invention

Graph 1. Plasma concentration versus time profile of f-152 in mouse

(I.V. administration, 10 mg/kg)



f-152



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Graph 2. Plasma concentration versus time profile of ER-803064 in mouse
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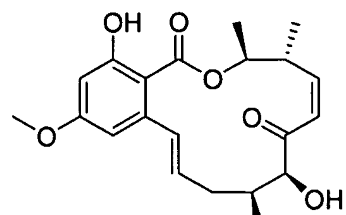

ER-803064

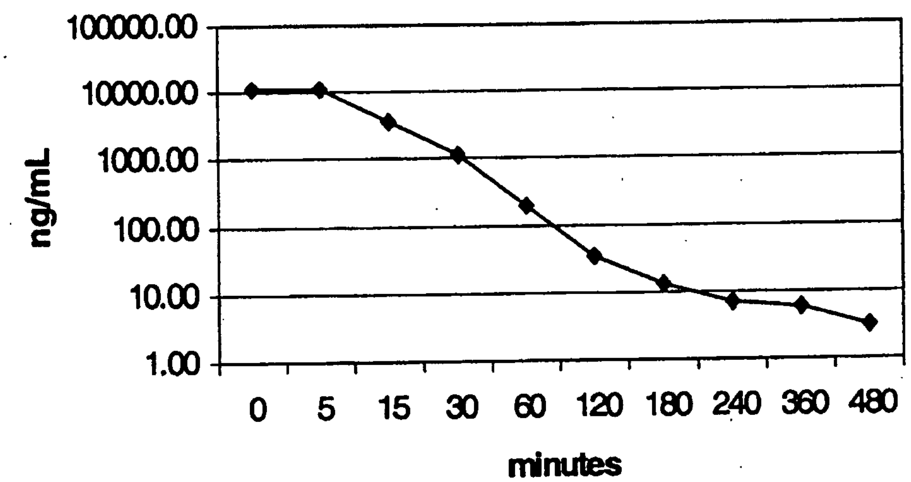

Exhibit 4

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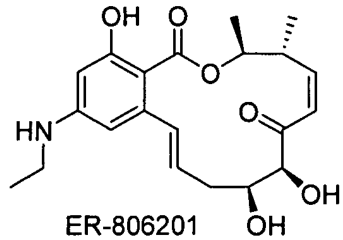
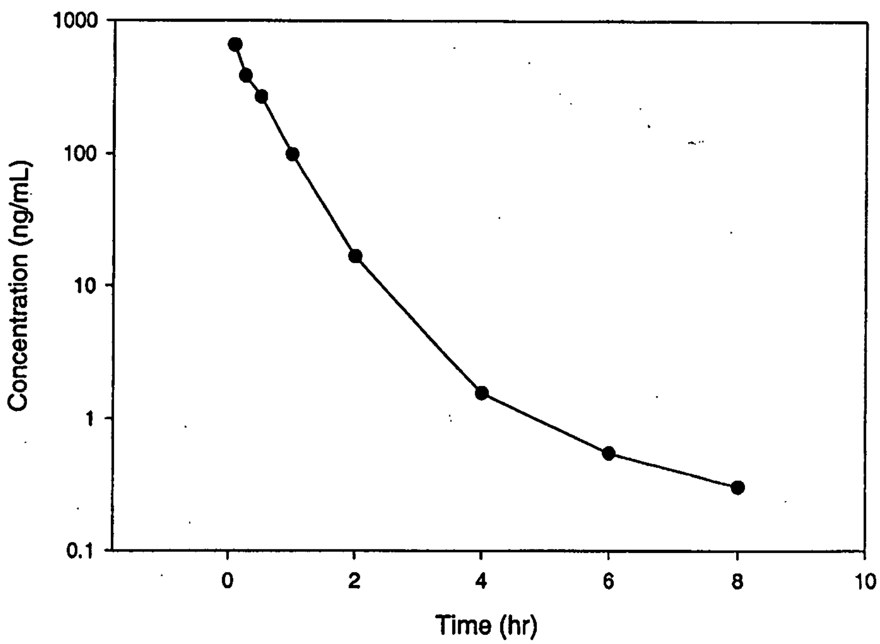
Graph 3. Plasma concentration versus time profile of ER-806201 in mouse

(I.V. administration, 3 mg/kg)



ER-806201



- EXHIBIT 5 -

Copies of references cited in section 7 of Dr. John (Yuan) Wang's Declaration

1. Ghosh *et al.*, "NF-κB and REL Proteins: Evolutionary Conserved Mediators of Immune Responses" *Annu Rev Immunol.* **1998,** *16,* 225-260.

2. Drouet *et al.,* "Enhancers and Transcription Factors Controlling the Inducibility of the Tumor Necrosis Factor-α Promoter in Primary Macrophages" *J. Immunol.* **1991,** *147(5),* 1694-1700.

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Annu. Rev. Immunol. 1998. 16:225–60

NF-κB AND REL PROTEINS: Evolutionarily Conserved Mediators of Immune Responses

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KEY WORDS: NF-κB, IκB, signal transduction, transcription, gene expression

ABSTRACT

The transcription factor NF-κB, more than a decade after its discovery, remains an exciting and active area of study. The involvement of NF-κB in the expression of numerous cytokines and adhesion molecules has supported its role as an evolutionarily conserved coordinating element in the organism's response to situations of infection, stress, and injury. Recently, significant advances have been made in elucidating the details of the pathways through which signals are transmitted to the NF-κB:IκB complex in the cytosol. The field now awaits the discovery and characterization of the kinase responsible for the inducible phosphorylation of IκB proteins. Another exciting development has been the demonstration that in certain situations NF-κB acts as an anti-apoptotic protein; therefore, elucidation of the mechanism by which NF-κB protects against cell death is an important goal. Finally, the generation of knockouts of members of the NF-κB/IκB family has allowed the study of the roles of these proteins in normal development and physiology. In this review, we discuss some of these recent findings and their implications for the study of NF-κB.

INTRODUCTION

The immune system responds to enormous diversity when mounting a defense. It is surprising therefore that relatively few signaling systems have been identified that directly perform the complicated task of communicating states of injury and infection. One way in which such signaling is accomplished is via

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inducible messengers such as cytokines, soluble molecules that are generally produced by and act on immune cells. Some cytokines induce the proliferation and differentiation of specific cells, for example, the effect of IL-2 on T cells and of GM-CSF and G-CSF on macrophages and granulocytes. Other cytokines (e.g. IL-1, IL-6, IL-8, and TNFα) induce the acute phase response in inflammation or protect uninfected cells from viral infection (interferon-β). Cytokine signaling to B cells results in their differentiation into plasma cells that then produce antibodies. It is therefore intriguing that in each of these different systems one common transcriptional activator, known as NF-κB, is targeted as an integral messenger in the enhancement of the response.

NF-κB is a eucaryotic transcription factor that exists in virtually all cell types. It was first described in 1986 as a nuclear factor necessary for immunoglobulin kappa light chain transcription in B cells (hence the name, nuclear factor-κ B) (1). In mature B cells and plasma cells, NF-κB is localized to the nucleus where it binds a 10 base-pair region of the kappa intronic enhancer and activates transcription. It was originally thought that NF-κB was not produced in other cells, including pre-B cells, because it could not be detected in these cell types by a sensitive gel-shift assay using the Igκ DNA-binding site. Subsequently however, it was found that the DNA-binding ability of NF-κB in these cells was masked by the presence of an inhibitor (2, 3). It is now known that NF-κB preexists in the cytoplasm of most cells in an inactive form bound to the inhibitor, IκB. Upon receipt of an appropriate signal, NF-κB is released from IκB and translocates to the nucleus where it can upregulate transcription of specific genes. It is critical to note that NF-κB's ability to respond to a signal makes it an inducible factor, and the fact that NF-κB activity does not require new protein synthesis allows the signal to be transmitted quickly. Furthermore, NF-κB communicates with both the cytoplasm and the nucleus and is therefore able to carry messages directly to their nuclear targets.

Since the discovery of NF-κB, its responsive sites (κB sites) have been characterized in the promoters and enhancers of numerous genes, making NF-κB an important component in the inducible expression of many proteins, including cytokines, acute phase response proteins, and cell adhesion molecules (4, 5). A closer inspection of these genes reveals a common purpose, namely, enhancement of the immune response. In addition to these inducible genes, NF-κB has been found in an active, nuclear form in mature B cells, plasma cells, macrophages, and some neurons. Because differentiation of cells is defined by the production of cell-type specific proteins, the constitutive activation of NF-κB probably allows some cells to maintain a differentiated phenotype. In this review we describe the known members of the NF-κB family of transcription factors, recent knowledge about the signaling pathways leading to their activation, and their potential role as central coordinators of the innate immune response.

THE NF-κB/REL AND IκB PROTEINS

The NF-κB Signaling System Is Evolutionarily Conserved

Nature has not overlooked the elegance of the NF-κB signaling system. Indeed, this system has been conserved to operate on divergent genes in many different species (6). In Drosophila three NF-κB molecules have now been described—*dorsal, dif,* and *relish* (7–9). The first Drosophila rel protein described was the dorsal/ventral morphogen, Dorsal, which delineates polarity by specifically activating genes on one side of the Drosophila embryo and repressing genes on the other (10). The signal activating Dorsal passes through a transmembrane protein called Toll, which bears a remarkable likeness to the cytoplasmic domain of the type I IL-1 receptor (11) and passes through a serine/threonine protein kinase, pelle, which is also homologous to IRAK (IL-1 receptor-associated kinase) from mammalian cells (12, 13). Dorsal, like NF-κB, is retained in the cytoplasm by an inhibitory protein called cactus (14, 15). The release of Dorsal from cactus (16) requires the kinase pelle, and although the exact sequence of events has not been determined, Dorsal itself is also phosphorylated (12, 17, 18). Interestingly, a κB-dependent mouse enhancer can operate successfully in transgenic flies to direct transcription of the Dorsal-responsive gene, *rhomboid*, an experiment that succinctly illustrates the versatility of the entire Dorsal/NF-κB transcription system, which has been adapted for use on a variety of genes (6).

A more striking similarity in function is observed in the case of the the second rel protein described in Drosophila, known as dif (8). Dif appears to be the fly equivalent of the inducible form of NF-κB because it is active only under certain circumstances and upregulates transcription of insect immunity genes. Dif preexists in the fat body of insects, an organ that is thought to be the ancestral equivalent of the liver in vertebrates (19). Interestingly, the liver is the center for production of the acute phase proteins in the acute phase response to inflammation, a condition partly regulated by NF-κB (20). Dif becomes activated as a result of infection; the dif-activated response is therefore part of a primitive innate immune system controlled by rel proteins (19). Although NF-κB sites have been found in the promoters of many of the insect antibacterial and antifungal genes, including *cecropin, attacin, defensin, diptericin, drosocin,* and *drosomycin*, a recent report suggests that not all the genes are regulated by Dorsal or Dif (21). The gene most dependent on Dorsal and Dif was that encoding the antifungal protein drosomycin, while the genes encoding the antibacterial proteins cecropin, attacin, and defensin were only partly dependent. These results suggested an additional signaling pathway in insects that is involved in responses to microbial infection (21). The third known member of the Rel-family in Drosophila is Relish, which appears to be the homolog of the mammalian p105 and p100 proteins (9), with the exception that it has

a serine-rich region in the place of the glycine-rich region (see below). This protein was reported recently, and as yet very little is known about its regulation except that it is highly upregulated upon bacterial infection. Nevertheless, its similarity to p105 and p100 once again reinforces the striking evolutionary conservation of the Rel/NF-κB family.

An intriguing parallel to the Toll/Dorsal/Dif system exists in plants. The product of the plant N gene is homologous to Toll and the IL-1R (22). This gene encodes a transmembrane receptor that confers resistance to tobacco mosaic virus infection probably by signaling the upregulation of the pathogenesis resistance proteins. Although an NF-κB-like protein has not been described in plants, the N protein may ultimately communicate with a similar transcription factor enabling the plant to activate an innate type of immune system. Recently, however, the gene encoding the NIM1 (noninducible immunity) gene in Arabidopsis, which plays a critical role in systemic acquired resistance and disease resistance in plants, has been cloned and found to be homologous to IκB (23). The signaling system of Toll and Dorsal/Dif also led to the search for an equivalent system in mammals. It is gratifying that a mammalian Toll homolog has now been identified, that appears to be able to induce the expression of cytokines and genes known to play an important role in innate and adaptive immunity (24). Therefore, Toll appears to function in vertebrates as a nonclonal receptor that modulates the immune response by activating NF-κB. These findings also further strengthen the notion that signaling in disease resistance responses from flies to mammals utilizes an ancient, evolutionarily conserved, and ubiquitous pathway that involves the Toll/NF-κB/IκB family of proteins.

Structure of NF-κB

NF-κB is a dimer of members of the rel family of proteins (reviewed in 4, 5, 25). Each family member contains an N-terminal 300 amino acid conserved region known as the rel homology domain (RHD). This region is responsible for DNA-binding, dimerization, and interaction with IκB family members. It also contains a nuclear localization sequence. The first NF-κB molecule described was a heterodimer of p50 and p65 subunits (5, 25). This protein is still what is commonly referred to as NF-κB despite the diversity of other members now known. Because the two rel proteins making up the complex each contact one half of the DNA binding site, the slight variations possible in the 10 base pair consensus sequence, $^{5'}$GGGGYNNCCY$^{3'}$, confer a preference for selected rel combinations (26). Other rel members include Dif, Dorsal, and Relish (Drosophila, see above), v-rel (chicken oncogene), c-rel, p52, and rel-B (see Figure 1) (reviewed in 4, 5, 25). The transcription factor NFAT (nuclear factor of activated T cells) is also homologous in the Rel-homology domain and is therefore sometimes considered to be a member of the Rel-family of proteins (27). In addition to showing specificity for DNA binding sites, each combination of

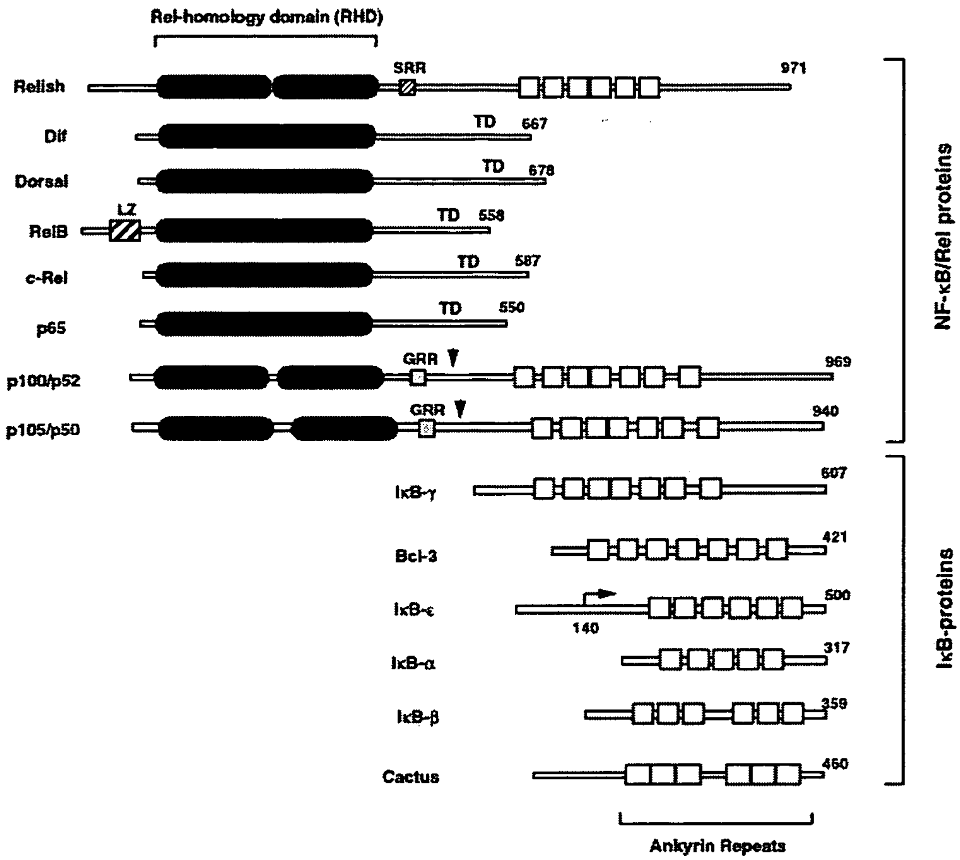

Figure 1 The Rel/NF-κB/IκB family of proteins. Members of the NF-κB/rel and I-κB families of proteins are shown. The number of amino acids in each protein is shown on the right. The *arrows* point to the endoproteolytic cleavage sites of p100/p52 and p105/p50: RHD, rel homology domain; TD, transactivation domain; LZ, leucine zipper domain of rel-B; GRR, glycine-rich region; SRR, serine-rich region in Relish. IκB-ε has been proposed to be translated from either the first methionine or from an internal methionine at position 140 (69).

rel proteins also has its own transactivating potential (25). Although most of the NF-κB proteins are transcriptionally active, some combinations are thought to act as inactive or repressive complexes. Thus, p50/p65, p50/c-rel, p65/p65, and p65/c-rel are all transcriptionally active, whereas p50 homodimer and p52 homodimer are transcriptionally repressive (28–32). Because p50 and p52 lack a variable C-terminal domain found in the activating rel proteins, this domain is most likely responsible for transactivation of NF-κB-responsive genes.

The first glimpse of how proteins of the Rel-family bind to DNA came from the crystal structure of p50 homodimers bound to a κB site (33, 34). Each p50 molecule is comprised of two domains joined by a flexible link. The core of these domains is made up of β-strands that fold in a pattern similar to immunoglobulin domains, although the significance of this similarity is unknown. Dimerization occurs exclusively via the C-terminal domains where a set of hydrophobic side chains interdigitate, clamping these regions together. In both immunoglobulin-like domains, β-strands are connected by loops that are responsible for an unusual DNA-contact interface. Both N- and C-terminal regions are involved in DNA binding, and the dimerization and DNA-binding contacts of the C-terminal domain form a novel continuous interface that essentially "locks" NF-κB to the major groove. This DNA-binding method is also noteworthy in the extensive number of contacts made by the loops to both the sugar-phosphate backbone and the bases. Mutational analysis has confirmed the importance of the bases involved in these interactions (35–37). The overall structure of the p50 dimer forms a molecule likened to a butterfly with the DNA binding site gripped tightly at its center and the immunoglobulin-like domains forming "wings" at the periphery (33, 38). The structure of the p65 RHD bound to DNA has also been solved recently (39). Although the overall architecture of the complex is quite similar to that of p50, the p65-DNA complex reveals a few novel features. Unlike the p50 homodimer, p65 subunits are not symmetrically arranged on the DNA target. While one of the subunits binds a four base-pair DNA half site with sequence specificity, the other subunit displays a remarkably different mode of DNA recognition, retaining only partial specificity for the other half site. This novel mode of DNA recognition exhibited by p65 probably helps to explain the wide range of DNA sequences recognized by NF-κB. Finally, the solution structure of the NFAT DNA-binding domain has also been determined (40). Unlike the other Rel-proteins NFAT-binds to DNA with the AP-1 transcription factor, and the structure of the NFAT-RHD reveals how this interaction might occur. The insert region in NFATc most probably interacts with c-jun in the NFAT-AP-1 complex, and in keeping with this model, mutations in the insert region reduce cooperativity between NFAT and AP-1 without affecting DNA binding by NFATc (40).

The structure of NF-κB p50 and p65 has also helped in understanding how NF-κB interacts with other regulatory proteins. In particular, the structure of p50 bound to DNA explains how NF-κB interacts with the HMG-I(Y) protein, in the transcription factor complex formed on the enhancer of the β-interferon gene (41). One of the sites for HMG-I(Y) is in the middle of a NF-κB binding site where the HMG-I(Y) protein contacts DNA from the side of the minor groove (41). The NF-κB p50 dimer approaches the DNA from the side of the major groove, and the structure of the p50-DNA complexes shows that the side

of the minor groove is empty (33, 34). Therefore, the binding site for HMG-I(Y) remains accessible from the minor groove. The two helices in the N-terminal domain of p50 are close to this region and hence affect the accessibility to the minor groove. Since the sequence corresponding to these helices is variable in different Rel-members and is significantly shorter in p65, it is possible that the binding of HMG-I(Y) could be facilitated when κB sites are bound by other combinations of Rel proteins, such as p50:p65.

Biogenesis of NF-κB p50 and p52

The mRNA retrieved from the cloning of p50, surprisingly, was found to encode for a much larger protein of approximately 105 kDa (42, 43). Likewise, the mRNA for p52 also encodes a protein of 100 kDa (44–46). The N-terminal regions of p105 and p100 are identical to the p50 and p52 proteins, respectively (see Figure 1). Inspection of the C-terminal portion of these molecules revealed several ankyrin repeats, reminiscent of the ankyrin repeats found in the NF-κB inhibitor molecule, IκB (discussed below). Later it was demonstrated that p100 and p105 are posttranslationally cleaved to produce the smaller p50 and p52 products (47–50). This processing is ATP-dependent and requires polyubiquitination in vitro (48, 51). Furthermore, because cytosolic fractions enriched with proteasomes support the processing of p105 in vitro, and proteasome inhibitors block processing in vitro and in vivo, the proteasome complex is implicated in the degradation of the C-terminal region of p105 (51, 52). The exact reason for translation of the larger product is unknown; however, the processing event probably represents a further level of control of the activation of NF-κB. The longer p105 and p100 proteins are exclusively cytosolic because of the masking of the nuclear localization signals on p50 and p52 by the C-terminal ankyrin-repeat region (53). Stimulation of cells with agents that activate NF-κB causes an increase in the rate of degradation of the C-terminal region of these proteins, and consequently increased amounts of p50 and p52, complexed with p65, are produced (54).

The complete model of the generation of p50 is, however, far from complete. Recent work suggests that there may be two pathways for the processing of p105: a signal-dependent pathway and a signal-independent pathway (55). The processing described above may be relevant for p105 bound to p65 in the context of a signal-dependent event; that is, upon stimulation, degradation of the C-terminal region of p105 increases, allowing this "pool" of NF-κB to translocate to the nucleus. Indeed, a phosphorylation event on the C-terminal of p105 is thought to be required for this processing (56). However, a preexisting pool of p50 is also associated with p65 and IκBα, which is generated constitutively in the absence of signal (see below). The processing event for the generation of this p50 appears to be regulated by a fascinating novel mechanism

(55). A 23–amino acid glycine-rich region (GRR) within the N-terminal of p105 is necessary and sufficient for directing the cleavage of p105 a short distance downstream from this region. Mutation or deletion of the C-terminal region (the target for the phosphorylation and degradation of p105 in the signal-dependent pathway) appears to have no effect on the generation of p50 in both unstimulated transfected COS cells and in vitro. Furthermore, cleavage also occurs when this glycine-rich sequence is inserted into a heterologous protein. The proteolytic event only occurs C-terminal to the GRR; however, the exact cleavage site is not determined by a specific linear distance from the glycine-rich region.

Studies carried out on p100 indicate that a corresponding GRR also plays an analogous role in directing its cleavage. However, the p100 GRR has not been extensively characterized (57). Although it is not yet clear whether a specific GRR-protease exists, current evidence already hints at a fascinating process whereby the presumed "GRR-protease" recognizes the GRR but cleaves downstream of its binding site in a sequence-independent manner. In some ways, this process is similar to that observed in case of Type III–restriction endonucleases (e.g. Fok I) (58). Clearly, however, determination of the mechanism by which GRR-mediated cleavage of p105 is followed by the rapid degradation of the C-terminal fragment, presumably by the proteasome, will be crucial to reaching a fuller understanding of how p105 and p100 are processed.

IκB Proteins: Inhibitors of NF-κB Activity

Activation of NF-κB is regulated by its cytoplasmic inhibitor, IκB. IκB binds NF-κB and masks its nuclear localization signal, thus retaining it in the cytoplasm (25, 59). Like NF-κB, IκB is a member of a larger family of inhibitory molecules that includes IκBα, IκBβ, IκBε, IκBγ, and Bcl-3 in higher vertebrate cells and cactus in Drosophila (see Figure 1). All of these inhibitors contain multiple regions of homology known as the ankyrin-repeat motifs. The ankyrin repeats are regions of protein/protein interaction, and the specific interaction between ankyrin repeats and rel-homology domains appears to be a crucial, evolutionarily conserved feature of the regulation of NF-κB proteins. Each IκB differs in the number of ankyrin repeats, and this number appears to influence the specificity with which IκB pairs with a rel dimer. As mentioned above, p100 and p105 also contain ankyrin repeats and are sometimes included in the IκB family.

The best-characterized IκB is IκBα, mainly because it was the first member of this family to be cloned (60, 61). IκBα is a 37-kDa protein that has a tripartite organization also seen in IκBβ: an N-terminal domain that is phosphorylated in response to signals, a central ankyrin repeat domain, and a C-terminal PEST domain that is involved in the basal turnover of the protein (25). As is discussed later, most of the current knowledge about signaling through NF-κB derives

from studies carried out on this protein. Physiologically the defining characteristic of IκBα is its ability to regulate rapid but transient induction of NF-κB activity, owing to the participation of IκBα in an autoregulatory feedback loop; that is, the activation of NF-κB causes the upregulation of transcription of IκBα, which serves to shut off the signal (62–65). This upregulation occurs owing to the presence of κB sites in the IκBα promoter (66, 67). Thus IκBα is thought to maintain the transient effect of inducing agents on the transcription of NF-κ B responsive genes. The continuing presence of certain inducing agents (for example, LPS) however causes NF-κB to be maintained in the nucleus despite the upregulation of IκBα mRNA synthesis, and this persistent activation of NF-κB is regulated by IκBβ (68).

IκBβ is a 45-kDa molecule that was cloned later and hence is less well characterized than IκBα (68). Both the biochemical purification and immunoprecipitation studies indicate that the majority of p50:p65 and p50:c-Rel complexes are regulated by IκBα and IκBβ (68, 69). It was originally anticipated that IκBβ would prefer different combinations of rel proteins than IκBα allowing a divergence in the signal transduction pathway (70). Surprisingly, IκBβ binds the same rel subunits as IκBα; the divergence appears to be at the level of the incoming signal and at the timing of the onset and duration of the response (68). Both IκBα and IκBβ are rapidly degraded after cells are treated with the inducing agents IL-1 and LPS; however, IκBα is retranscribed as a result of NF-κB activation, whereas IκBβ is not. Thus, IκBβ levels remain low until the NF-κB activating signal is attenuated. The process for IκBβ degradation is less well understood and, as discussed later, appears to be more complex than that for IκBα.

The most recent member of the IκB family to be described is IκBϵ. Unlike IκBα and IκBβ, which were identified and characterized during purification of NF-κB:IκB complexes from cells, IκBϵ was identified as a protein that specifically interacted with p52 in a yeast two-hybrid assay (69). Surprisingly, subsequent characterization of the protein revealed that it was unable to interact with either p50 or p52; instead it appeared to be a specific inhibitor of p65 and c-Rel complexes. Immunoprecipitation experiments revealed that in vivo IκBϵ was associated exclusively with p65:p65 and p65:c-Rel complexes (69). IκBϵ can be degraded with slow kinetics upon stimulation of cells with the majority of inducers of NF-κB, and as with the other IκBs, this degradation requires two N-terminal serine residues. At present it seems that IκBϵ acts as a specific inhibitor of a subset of Rel complexes, and it therefore probably regulates the expression of specific genes, e.g. IL-8, whose promoters bind preferentially to p65 and c-Rel complexes (71).

Bcl-3 is the most unusual member of the IκB family. It was first identified by cloning a t(14:19) chromosomal breakpoint from a B cell chronic lymphocytic

leukemia (72). Structurally, Bcl-3 is very much like the other IκB family members in that its predicted protein structure contains 7 ankyrin repeats. However, unlike IκBα and IκBβ which are located in the cytoplasm, Bcl-3 appears to be located in the nucleus and has a binding specificity for p50 and p52 homodimers (73–77). Although Bcl-3 may function as a transactivator by removing these inhibitory NF-κB complexes from κB sites, p52:Bcl-3 complexes can also directly bind to κB sites and activate transcription (78). Thus, Bcl-3 is apparently unique among the IκB proteins in that its binding to rel proteins leads to transcriptional activation rather than repression. How Bcl-3 fits into the scheme of rel protein regulation remains to be defined, although the recent knockout of Bcl-3 will probably help in this regard (see below).

Finally, very little is known about the IκB family member IκBγ, which is a 70-kDa molecule detected only in lymphoid cells (79). Its sequence is identical to the IκB-like C-terminal region of p105, and indeed IκBγ is the product of an alternate promoter usage that produces an mRNA encoding the C-terminal portion of p105. Although the initial report of IκBγ suggested that it functioned as a *trans*-inhibitor of Rel-proteins, similar to the other IκB proteins (79), subsequent studies have suggested that IκBγ probably plays a more limited role, probably inhibiting only p50 or p52 homodimers (80). The very narrow cell type distribution of this protein, namely, only in mature B cell lines from mice (42, 79), also hints at a very specialized function. The precise role of IκBγ in regulating Rel-proteins remains to be elucidated.

INDUCIBLE ACTIVATION OF NF-κB

NF-κB Is Induced by Many Different Inducers and Affects Many Different Genes

One of the most interesting aspects of NF-κB is the variety and the nature of the inducers that lead to its activation. As mentioned above, the activation of NF-κB is achieved primarily through the degradation of IκB proteins. Many agents when added to cells will induce the activation of NF-κB (see Figure 2). Each signals to the cell that damage (reactive oxygen intermediates, uv light) or infection (LPS, viral transactivating proteins, double-stranded RNA) has occurred. Some inducers are themselves by-products of a signal transduction cascade (IL-1, TNFα, sphingomyelin, products of membrane turnover, and calcium ionophores) (reviewed in 4, 5). Ultimately the signal is passed on to an IκB kinase (see below), which phosphorylates IκB and leads to its degradation. NF-κB is also responsive to reactive oxygen intermediates (ROIs). Since ROIs are sometimes released and produced during the inflammatory response, this pathway for NF-κB activation may be physiologically relevant. The activation of NF-κB by ROIs, the tax protein, and other known inducers can be

inhibited by reducing agents and ROI scavengers, including dithiocarbamates and N-acetyl-L-cysteine (81). These data suggest that the redox state of the cell may be a general mechanism for upregulating NF-κB responsive gene transcription.

The nature of the signals that lead to the activation of NF-κB strongly suggests that NF-κB plays a critical role in innate immune responses (5). NF-κB plays such a role in the activation of immune cells by upregulating the expression of many cytokines essential to the immune response [for an extensive list, see Kopp & Ghosh (5) and see Figure 2]. In particular, NF-κB stimulates the production of IL-1, IL-6, TNFα, lymphotoxin, and IFN-γ. Furthermore, some of these cytokines, e.g. IL-1 and TNFα, activate NF-κB themselves, thus initiating an autoregulatory feedback loop. All of these cytokines have multiple effects that contribute to inflammation (82). NF-κB is an important protein in the regulation of the acute phase response of inflammation, which is the systemic defense established to restore homeostastis after infection or injury. The clinical hallmark of this response is the rapid production of acute phase response proteins by the liver, a response that can be measured in the bloodstream. Many of the acute phase proteins have κB sites in their promoters or enhancers and are produced as a result of exposure of cells to the inflammatory cytokines IL-1 and IL-6. These include serum amyloid A protein 1, α1 acid glycoprotein, angiotensinogen, complement factor B (Bf), and the C3 component of complement (4, 5). It is interesting to note that anti-inflammatory drugs such as the salicylates and the corticosteroid dexamethasone inhibit NF-κB activation in cultured cells, suggesting that NF-κB may be a good target for potential therapies against chronic inflammatory diseases (83–86). Understanding the mechanism by which inducers of NF-κB transduce their signals to the cytosolic NF-κB:IκB complex is a major area of investigation at present. Below we summarize some of the recent advances in this area.

Signal-Induced Degradation of IκBα

Two lines of evidence pointed toward phosphorylation of IκBα as a means of activating NF-κB. First, the phorbol ester PMA, which is known to act through the kinase PKC, could activate NF-κB in intact cells (87, 88). Second, in vitro, IκBα responds to treatment with various kinases (PKC, PKA, and HRI) by releasing NF-κB (89). Subsequently, it was demonstrated that in vivo degradation of IκBα was required for the appearance of NF-κB in the nucleus (49, 62, 90–92). In addition, some investigators were able to demonstrate a slower migrating form of IκBα by immunoblot analysis in extracts from stimulated cells treated with protease inhibitors (49, 51, 93, 94). This slower moving IκBα could be abolished by treatment with phosphatase, which also implied that IκBα is phosphorylated before degradation. The question remained whether

the phosphorylated IκB was degraded while it was still part of the NF-κB:IκB complex or whether it first dissociated from NF-κB? This was answered by co-immunoprecipitating rel proteins with IκBα, from stimulated cells treated with proteasome inhibitors to block the degradation of phosphorylated IκBα, which demonstrated that IκBα undergoes proteasome-mediated degradation after phosphorylation but before dissociation (90, 93–95).

Protein degradation within cells is a very tightly regulated multistep process, believed to be initiated by site-directed ubiquitination of target proteins. Initially it was thought that ubiquitination-dependent protein degradation was used exclusively for turnover of misfolded or defective proteins (96, 97). However, subsequent studies demonstrated that modulation of the level of some regulatory proteins, such as the periodic degradation of cyclins, was mediated through this mechanism. The actual degradation of ubiquitinated proteins is carried out by the multicatalytic ATP-dependent proteasome complex. The first evidence that inducible degradation of IκBα might be dependent on ubiquitination was provided by experiments using peptide aldehyde inhibitors of the proteasome such as calpain inhibitor I (ALLN), PSI, and MG-132 (Z-LLF-CHO) (51, 93–95). Treatment of cells with these inhibitors blocked the degradation of IκBα after stimulation with NF-κB inducers, and phosphorylated IκBα accumulated in the cytoplasm. It was determined by several groups that inducible phosphorylation on IκBα occurs on two N-terminal serines positioned at residues 32 and 36 (98–101). Mutation of these residues by site-directed mutagenesis blocked phosphorylation of IκBα in response to activating signals and thereby prevented subsequent degradation of the protein. The use of proteasome inhibitors also allowed the direct mapping of the phosphorylation sites on ser-32 and 36 of IκBα (102). Similar approaches also led to identification of ubiquitinated IκBα, and

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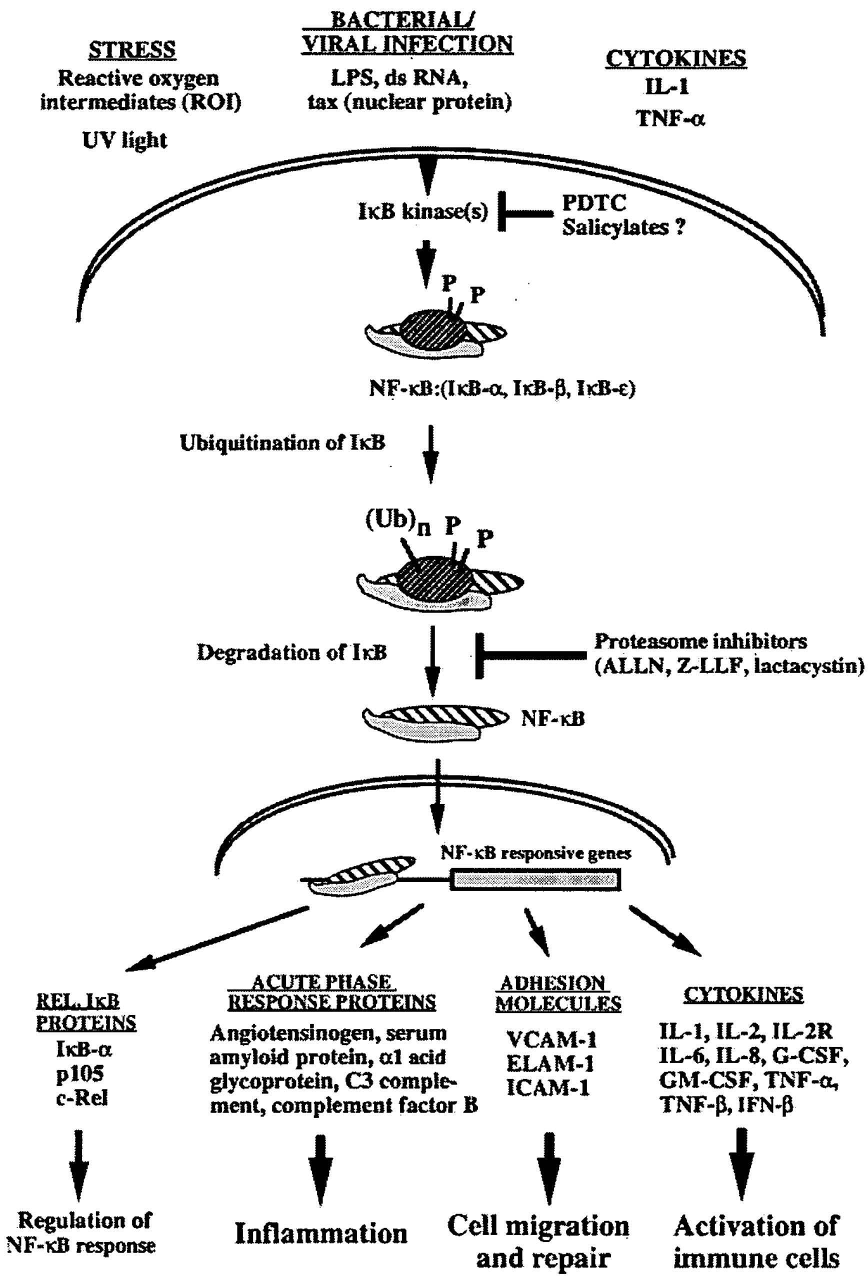
Figure 2 Proposed mechanism for induction of NF-κB proteins. NF-κB molecules exist in the cytoplasm of most cells in an inert, non-DNA-binding form. Some NF-κB homo- and heterodimers are retained in the cytosol by the inhibitory molecules, IκBα, IκBβ, and IκBϵ. The NF-κB precursor proteins p105 and p100 are also retained in the cytoplasm as heterodimers with other rel proteins by virtue of their IκB-like C-terminal regions (54, 171). Presumably the ankyrin repeats in the C-terminal regions of p105 and p100 fold back to mask the nuclear localization sequence of p65. Extracellular inducers creating stress or indicating infection cause the activation of IκB kinase(s) through poorly defined pathway(s). This process can be blocked by antioxidants (e.g PDTC) and salicylates. Activation of NF-κB occurs predominantly through the phosphorylation and degradation of IκB proteins. Phosphorylation of IκB proteins is followed by polyubiquitination, and they are degraded as part of their respective ternary complexes. This degradation can be inhibited by inhibitors of the proteasome (ALLN, Z-LLF, lactacystin). "Free" NF-κB translocates to the nucleus, where it upregulates expression from many genes involved in the immune and inflammatory responses.


STRESS
Reactive oxygen intermediates (ROI)
UV light
BACTERIAL/ VIRAL INFECTION
LPS, ds RNA, tax (nuclear protein)
CYTOKINES
IL-1
TNF-α
IκB kinase(s)
PDTC
Salicylates ?
P
P
NF-κB:(IκB-α, IκB-β, IκB-ε)
Ubiquitination of IκB
(Ub)n
P
P
Degradation of IκB
Proteasome inhibitors (ALLN, Z-LLF, lactacystin)
NF-κB
NF-κB responsive genes
REL, IκB PROTEINS
IκB-α
p105
c-Rel
ACUTE PHASE RESPONSE PROTEINS
Angiotensinogen, serum amyloid protein, α1 acid glycoprotein, C3 complement, complement factor B
ADHESION MOLECULES
VCAM-1
ELAM-1
ICAM-1
CYTOKINES
IL-1, IL-2, IL-2R
IL-6, IL-8, G-CSF,
GM-CSF, TNF-α,
TNF-β, IFN-β
Regulation of NF-κB response
Inflammation
Cell migration and repair
Activation of immune cells

multiple ubiquitin molecules were found to covalently conjugate to two neighboring N-terminal lysine residues at positions 21 and 22 (95, 103). Taken together, these experiments revealed the following order of events required for NF-κB activation: 1. stimulation of cells with inducers of NF-κB results in phosphorylation of IκBα at positions ser-32 and ser-36; 2. phosphorylated IκBα is then ubiquitinated on lys-21 and lys-22; and 3. this triggers the rapid degradation of the protein by the 26S proteasome. Neither phosphorylation nor ubiquitination alone is sufficient to dissociate the NF-κB:IκBα complex; hence, free NF-κB is only released after degradation of IκBα. Intriguingly, a structural motif similar to that required for IκB phosphorylation and ubiquitination has been identified in the adhesion complex protein β-catenin (104). Mutagenesis experiments have demonstrated that this motif is involved in the phosphorylation and degradation of β-catenin and is therefore required for the regulation of the steady-state levels of the protein (104). Therefore, these findings indicate that the multistep mechanism of phosphorylation, ubiquitination, and degradation may also be used for the regulation of other proteins.

The IκB Kinase

Identification of the critical role played by specific phosphorylation of serines 32 and 36 in the initiation of IκBα degradation has prompted a concentrated search for the so-called IκBα kinase. A number of different kinases, including PKC-ζ, PKA, raf-1, double-stranded RNA dependent kinase (PKR), and p90-ribosomal S6 protein kinase (p90-RSK), have been suggested to fulfill this role, and most of these have been shown to cause dissociation of the NF-κB:IκBα complex in vitro (89, 105–108). However, besides p90-RSK, which appears to phosphorylate only Ser 32 (108), none of these kinases is able to specifically phosphorylate both of the N-terminal serine residues of IκBα required for activation in vivo. Mutational studies have revealed that substitution of the serine residues with threonines also prevents inducible phosphorylation of IκBα, suggesting that a completely novel, exquisitely serine-specific kinase is required (102). Furthermore, single amino acid substitution of either of these serines is sufficient to prevent NF-κB activation in response to numerous signals, including LPS, TNFα, and IL-1, demonstrating a critical requirement of the kinase for an intact substrate sequence (99, 102). Since most of the known inducers of NF-κB transmit their intracellular signals through distinct pathways, there must be a convergence point where all these pathways intersect. One likely candidate for this convergence point is therefore the kinase responsible for phosphorylating IκB and initiating its degradation.

Intense effort is currently underway in many laboratories to clone and characterize this putative IκB kinase, which until recently remained elusive. However, two recent reports have described the partial purification and characterization

of a high molecular weight (approximately 700 kDa) kinase complex from cytosolic extracts, which apparently specifically phosphorylates ser-32 and ser-36 of IκBα (109, 110). One unprecedented and entirely unexpected property of this kinase, besides its large size, is its apparent dependence on ubiquitination for activity (109). This conclusion is based upon observations made during the purification process, which suggested that separation of certain ubiquitin-conjugating molecules (e.g. Ubc4/Ubc5) from kinase-containing fractions inhibited its activity. Surprisingly ubiquitination does not appear to cause degradation of the kinase; instead it seems that the modification induces its activation by an as-yet-uncharacterized mechanism. Initially, it was reported that the kinase could be extracted in an active form from uninduced cells; however, it was later suggested that activation of the kinase was a consequence of stresses applied during preparation of the extracts (110). In keeping with this concept, when the kinase was extracted from cells by a rapid lysis procedure, it was found to be inactive, and its activity could be induced by ubiquitination (110).

This ability to isolate the IκBα kinase in an inactive form allowed investigation of potential upstream regulators of the kinase in addition to ubiquitination (110). Very little is known about the signals directly proximal to NF-κB activation, although several studies had previously demonstrated that TNFα-induced NF-κB activity could be mediated by activation of the protein kinase MEKK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase)-1 which lies in the signaling pathway leading from TNFα to activation of the mitogen-activated protein kinase JNK (c-jun N-terminal protein kinase) (111–113). Furthermore, the MEKK-1 to JNK signaling cascade can be activated by other known inducers of NF-κB such as UV irradiation and LPS, and upstream activators of MEKK-1 such as the small GTPases CDC42 and Rac-1 have also been shown to activate NF-κB (114). Together, these results suggest that MEKK-1 lies at the juncture of the pathways leading to JNK and NF-κB. Investigation of the role of MEKK-1 in this process revealed that a truncated form of the kinase could activate IκB kinase activity, although this activation differed slightly from that induced by ubiquitination. This difference was only manifested during an anion exchange chromatography stage of kinase purification in which the MEKK-1-activated IκB kinase eluted in a broader peak than the ubiquitination-activated complex. Hence it is possible that two similar but subtly different populations of the IκB kinase exist: one that is responsive to MEKK-1 and another that is activated by ubiquitination (110).

Curiously, another recent study has suggested that MEKK-1 does not play a role in NF-κB activation, a result clearly inconsistent with those described above (115). It is unclear why these different observations have been made; however, the recent identification of a MEKK-1-related kinase, termed NIK (NF-κB inducing kinase), which interacts with the TNF-receptor associated

factor (TRAF)-2 and stimulates IκBα degradation, supports the hypothesis that a MEKK-1-related kinase, rather than MEKK-1 itself, plays an important role in NF-κB activation (116). Indeed, a separate MEKK family member named MEKK-3 activates NF-κB, and although the mechanism of activation by MEKK-3 is unknown, it most likely functions in a manner analogous to MEKK-1, i.e. upstream of the IκB kinase (117). Interestingly, mutant forms of NIK block signaling from both TNF and IL-1 receptors, suggesting that the convergence of signaling pathways induced by these cytokines leading to NF-κB activation might occur upstream of the IκB kinase (116). However, these issues can be addressed only after molecular cloning and a full characterization of the IκB kinase.

A number of questions exist that can only be answered following thorough characterization of the IκB kinase. Principal of these is the issue of how different signaling pathways, whose known components are discrete, are able to activate the IκB kinase. A number of upstream activators of NF-κB have been identified, including members of the TRAF family of molecules (TRAF-2, -5, and -6) which can associate with several of the TNF receptor family of proteins (e.g. TNF-receptors type I and II, CD30, CD40, CD95, LT-β (118–124) and the IL-1 type I receptor (TRAF-6 only) (125). Furthermore, IL-1–induced NF-κB activation requires the accessory protein IL-1RAcP (IL-1 receptor-associated protein) (126, 127) as well as recruitment of a ser/thr kinase named IRAK (IL-1 receptor-associated kinase) (13). However, the convergence point for these signals is not yet known. If there is a single IκB kinase, then is it regulated by activator proteins that act as the end points of the various signaling pathways? Or do the different pathways merge further upstream before they activate the IκB kinase by, for example, activation of a kinase such as NIK, described above?

The apparent complexity of the IκB kinase itself poses a number of questions. Is this assembly of many proteins used only to activate NF-κB, or does it target other intracellular substrates? One possibility is that some of the subunits of the kinase are signaling and/or adapter molecules that can also serve distinct functions in separate systems. In this scenario, different subunits of the kinase complex might associate to allow interaction with different signaling pathways, and thus the kinase itself may be capable of responding directly to the many activating signals. It is therefore possible that the different subunits are targets for separate signaling cascades. For example, some may be the substrates for upstream kinases (e.g. MEKK-1), whereas others may be activated by ubiquitination or by association with small GTPases such as RhoA (114). More intriguing still is the possibility that the IκB kinase complex responds to incoming signals by generating an internal signaling cascade involving kinase and substrate subunits, which leads ultimately to activation of the IκB phosphorylating kinase.

Finally, a fascinating issue that remains unanswered is why this kinase was not recovered in the genetic screens in Drosophila? Several of the components of the pathway leading from the IL-1 receptor to NF-κB activation have Drosophila counterparts, e.g. Toll (IL-1 receptor), pelle (IRAK, mouse pelle-like kinase), Dorsal (NF-κB), and cactus (IκB), and therefore the absence of mutants of the IκB kinase seems surprising (128). However, this result would be expected if mutation in the kinase resulted in a lethal phenotype, suggesting that the kinase might be involved in regulating critical developmental processes. Cloning of the mammalian kinase will undoubtedly lead to identification of the Drosophila homolog and will provide answers to these questions.

Another major area of current interest is the mechanism by which phosphorylated and ubiquitinated IκB proteins are selectively degraded by the proteasome (96, 97). The current model hypothesizes that phosphorylation of the N-terminal serine residues of IκB in some way alters the conformation of the protein and exposes the lysine-containing sequences that are targeted by the ubiquitination machinery (25, 54, 59). One possible mechanism regulating selectivity for IκB molecules might be the existence of a specific ubiquitin ligase that recognizes defined substrate sequences present only in N-terminally phosphorylated IκB (96, 97). Attempting to identify an IκB-specific ligase is a major research objective for many laboratories. Lysine residues that are believed to be the sites of ubiquitination are conserved between the different IκB isoforms; however, mutation of these residues does not lead to a complete block of IκB degradation (69). The most likely explanation for this is that alternative lysines can be used for ubiquitination if the original sites are absent, although this has yet to be demonstrated. Phosphorylation and ubiquitination result in the IκB proteins adopting a conformation that is selectively recognized by the proteasome; it is possible that additional modifications such as phosphorylation of tyr-42 (see below) inhibit this recognition (129). Recent studies on the three-dimensional structure of the 20S proteasome from yeast have indicated that proteins destined for degradation must enter the structure through small openings (130). This suggests that IκB proteins are not degraded while in contact with NF-κB, but they are somehow stripped from NF-κB and threaded into the proteasome. However, identification of this mechanism must await three-dimensional structural analysis of the process.

Phosphorylation of IκBα at Other Amino Acid Residues

In addition to ser-32 and ser-36, IκBα is also phosphorylated at a number of other amino acid residues. The precise role of these phosphorylations is unclear at the moment, although we know phosphorylation of serines and/or threonines within the C-terminal PEST domain of IκBα affects the intrinsic stability of the protein (131, 132). The kinase responsible for this phosphorylation

has been identified as casein kinase II (CKII), which reportedly preferentially phosphorylates the serine residue at position 293 (131–134). CKII-mediated phosphorylation is constitutive and appears to be required for the turn-over of free IκBα but is not involved in the inducible degradation of IκBα in response to extracellular stimuli. The mechanism of this basal degradation has not yet been determined, nor has its precise role in NF-κB:IκB homeostasis, although answers to these questions will surely be forthcoming. It is interesting to note that the recently cloned IκBϵ lacks a PEST domain, suggesting that this may underlay differences in function between this and the other PEST-containing IκB isoforms (69).

Attention is also being paid to the potential role of tyrosine phosphorylation in the functional regulation of IκBα, and although two recent reports have addressed this issue, the results of these studies are somewhat conflicting (129, 135). In the first of these reports treatment of Jurkat-T cells with the potent tyrosine phosphatase inhibitor pervanadate (PV) led to tyrosine phosphorylation of IκBα coupled with activation of NF-κB (129). The site of this phosphorylation was tyr-42, and use of p56^{lck}–deficient Jurkat variants suggested that this kinase was responsible for the phosphorylation. More interesting, however, was the observation that tyr-42 phosphorylation resulted in dissociation of IκBα from NF-κB but not in its subsequent degradation. The conclusion drawn from this result was that tyrosine-phosphorylated IκBα is not recognized by the proteasome, and interaction with an SH2 domain-containing protein may be responsible for removal of IκBα from NF-κB. The subsequent report verified both the phosphorylation of tyr-42 and the lack of IκBα degradation in response to PV; however consistent with their prior observations, no accompanying activation of NF-κB was observed (135). In contrast, tyrosine phosphorylation of IκBα blocked NF-κB activation in response to TNF and this occurred via inhibition of the induced serine phosphorylation of IκBα. This finding suggests that phosphorylation of tyr-42 prevents phosphorylation of ser-32 and -36 by the IκB kinase, perhaps by altering the substrate structure required for recognition by the kinase. The reason for the conflicting data from these two studies is not clear at present, although differences between the cell types used in each may be a factor. Nevertheless, both reports indicate a crucial role for tyr-42 in IκBα regulation and suggest that either constitutive or induced control of this phosphorylation by kinases or phosphatases is fundamental to the function of IκBα. The lack of analogous tyrosine residues in either IκBβ or IκBϵ may contribute to differences in the regulation of these three IκB molecules, but further study of the role of tyr-42 is certainly required.

Differential Regulation of IκBα and IκBβ

The different IκB isoforms target different combinations of Rel-proteins; for example, IκBα and IκBβ associate predominantly with p50:p65 and p50:c-Rel

heterodimers, whereas IκBϵ interacts only with p65 and c-Rel hetero- and homodimers, and bcl-3 interacts with p50 and p52 homodimers (68, 69, 75). Different combinations of Rel-protein dimers preferentially bind to distinct DNA sequences (termed κB sites) and as such can differentially regulate gene expression (26). It is therefore relatively easy to understand how the regulation of separate IκB isoforms could differentially activate distinct Rel-protein dimers and control expression of individual genes. However, since the interaction specificity of IκBα and IκBβ appears to be indistinguishable, they are unlikely to function as differential activators of Rel protein dimers (68). Functional differences between the two isoforms only become apparent upon examination of the degradation of the proteins in stimulated cells.

All known inducers of NF-κB cause degradation IκBα. In contrast, in some cell types, IκBβ is only affected by a subset of inducers, e.g. LPS, IL-1, or the HTLV-1 tax protein, which cause a persistent activation of NF-κB varying in magnitude in a cell-type–specific manner (68, 136). This initially suggested fundamental differences in the regulation of IκBα and IκBβ; they were targeted by separate kinases induced by the distinct signaling pathways or ubiquitinated by distinct ubiquitin ligases. However, two N-terminal serine residues at positions 19 and 23 in IκBβ resembled Ser 32 and 36 in IκBα that are known to be critical for signal-induced degradation. Mutational studies indicated that Ser 19 and 23 in IκBβ were required for inducible degradation by inducers such as IL-1, LPS, and HTLV-1 tax (102, 136). In addition, other amino acids positioned close to these serines are also conserved between the two IκB isoforms. These data suggest that both IκBs are targeted by the same or very similar serine-specific IκB kinases and that the differences in their regulation patterns lie elsewhere. Degradation of IκBβ occurs with slower kinetics than does IκBα, and this may be due to a slower rate of phosphorylation of IκBβ, which may be a less efficient substrate for the IκB kinase (102). Therefore, inducers that only weakly activate the kinase may not provide a sufficient signal for IκBβ phosphorylation. Resolution of the nature of individual signals upstream of the IκB kinase will be required to test this hypothesis.

Despite the current consensus that both IκBα and IκBβ are targeted by the same or similar kinases, a few reports suggest that the regulation of IκBβ may differ more substantially from IκBα. The initial characterization of IκBβ suggested this isoform was unresponsive to TNF and PMA, but it now appears that the response of IκBβ to different inducers is dependent on the cell type used (137, 138). For example TNF leads to the degradation of IκBβ in human umbilical vein endothelial cells (HUVEC), whereas PMA can cause IκBβ degradation in certain 70Z/3 murine pre-B cell lines. These results strongly suggest that there does exist a distinct IκBβ kinase and that HUVECs and sublines of 70Z/3 differ from other cell lines such that the IκBβ kinase can be activated by TNF or PMA. However the fundamental assumption that IκBβ

is phosphorylated prior to degradation has been challenged in a recent report, which failed to detect changes in the phosphorylation status of IκBβ in stimulated vs unstimulated cells (138). Instead it appeared that serines 19 and 23 of IκBβ were constitutively phosphorylated even in the absence of any stimulus. Since Ser 32 and 36 of IκBα in these same cells are inducibly phosphorylated, the most likely explanation is that a single IκB kinase is constitutively active and continuously phosphorylates both IκB. The serines 32 and 36 of IκBα are kept in an unphosphorylated state by an IκBα-specific phosphatase in unstimulated cells, and inactivation of this phosphatase leads to phosphorylation and degradation of IκBα. However, the inducible degradation of IκBβ cannot be accounted for by such a model. The ability of phosphatase inhibitors such as okadaic acid and calyculin A to induce IκBβ degradation (139) does raise the possibility that a phosphatase is also involved in regulating the phosphorylation of IκBβ.

Recent work on IκBβ has focused on discerning the mechanism by which it mediates persistent activation of NF-κB. After activation, NF-κB causes upregulation of IκBα mRNA levels by binding to NF-κB sites in the IκBα promoter (25, 59). Newly synthesized IκBα then migrates to the cytoplasm where it can bind to and inactivate free NF-κB. However, during persistent NF-κB activation, some NF-κB is resistant to resequestration and retains the ability to enter the nucleus and initiate transcription. Following degradation of the initial pool of IκBβ, newly synthesized IκBβ, which is not basally phosphorylated, can complex with free NF-κB and prevent it from interacting with IκBα (140). Furthermore, unlike basally phosphorylated IκBβ, unphosphorylated IκBβ is not able to mask the NLS or DNA-binding domains of NF-κB (140). In this state IκBβ-bound NF-κB can still enter the nucleus and remain transcriptionally active (140, 141). Therefore, newly synthesized and unphosphorylated IκBβ appears to act as a chaperone for NF-κB and maintains its activity long after production of IκBα.

A recent report has also examined the differential regulation of NF-κB by IκBα and IκBβ from a different perspective. In this study the ability of the two IκBs to act as post-induction inhibitors of promoter-bound NF-κB was tested (141). While IκBα was a potent inhibitor of DNA-bound NF-κB, IκBβ was effective only in certain situations, e.g. when HMG-I(Y) was a part of the promoter complex. In addition this study demonstrated that, in a manner similar to LPS-induced pre-B cells, the persistently induced NF-κB in virus infected cells is a ternary complex with DNA and IκBβ (141).

Determining the mechanism by which unphosphorylated IκBβ is generated in persistently stimulated cells is crucial for further deciphering the regulation of IκBβ. During stimulation the appearance of the hypophosphorylated IκBβ can be blocked by cycloheximide, indicating that only newly synthesized IκBβ can become hypophosphorylated, and the phosphorylation status of the

already basally phosphorylated IκBβ is not affected (138, 140). The possible explanations for this would be either (i) the kinase responsible for basally phosphorylating IκBβ is inactivated in stimulated cells, or (ii) a phosphatase specific for IκBβ is activated or synthesized in stimulated cells. The recent identification of the sites of basal phosphorylation in the PEST-domain of IκBβ is therefore an important first step. Two serine residues at positions 313 and 315 are the critical residues whose phosphorylation status determines the ability of IκBβ to inhibit the DNA-binding activity of NF-κB (142). The kinase reponsible for phosphorylating these residues is casein kinase II, which is also responsible for basally phosphorylating IκBα (see above). Since the basal phosphorylation status of IκBα is not altered in stimulated cells, the most feasible hypothesis would involve an IκBβ-specific phosphatase that is either activated or newly synthesized in cells persistently stimulated with inducers of NF-κB activity.

Regulation of NF-κB Activation by PKA

The activity of NF-κB is primarily regulated by its sequestration in the cytosol through anchoring to the IκB proteins. Therefore, mechanisms leading to the disruption of the NF-κB:IκB complex and the subsequent translocation of NF-κB to the nucleus have received most attention. Nearly all other inducible transcription factors, including those that translocate from the cytoplasm to the nucleus, are themselves subjected to posttranslational modifications, which helps to regulate their activity. Recent studies indicate that the activity of NF-κB is also regulated by posttranslational modification, but the exact manner by which this occurs is unusual and involves cyclic-AMP (cAMP)-independent activation of PKA (143).

During purification of IκBβ from rabbit lung cytosol, it was noticed that a 42-kDa protein co-purified with IκBβ. This protein was subsequently identified as the catalytic subunit of PKA (PKAc), which is normally complexed with the regulatory subunit PKAr and requires cAMP for activation. However, PKAr was not found associated with the co-purified PKAc. Instead the PKAc could associate with the NF-κB:IκB complex via a bivalent interaction where the ATP-binding domain of PKAc interacts with either IκBα or IκBβ, while the substrate binding domain remains bound to the NF-κB p65 subunit. The association with NF-κB:IκB inhibits the PKAc catalytic activity, which can be induced upon stimulation of NF-κB activity via degradation of IκBα and -β. Activation of PKA by this mechanism does not therefore require cAMP and represents an entirely novel mode of activation for this kinase. But does this PKA influence the activity of NF-κB? Examination of the amino acid sequence of the p65 subunit of NF-κB reveals that it contains a consensus PKA phosphorylation site, and mutation of this sequence (at ser-276) inhibits the transcriptional activity

of NF-κB. Study of the possible targets of PKA-mediated regulation of NF-κB has demonstrated that catalytically active PKA does not significantly affect the dissociation of NF-κB:IκB complexes, the nuclear translocation of NF-κB, or the binding of NF-κB to DNA. Instead, PKA phosphorylation increases the transactivating activity of DNA-bound NF-κB.

An important issue raised by these studies is determining the mechanism by which phosphorylation by PKA alters the transcriptional activity of NF-κB. Recent results have demonstrated that cAMP response element binding protein (CREB)-binding protein (CBP) and the closely related factor p300 can interact with p65 and potentiate its transactivating ability (144, 145). Co-immunoprecipitation studies reveal that CBP/p300 can form a molecular bridge between DNA-bound p65, other transcription factors such as ATF-2 or c-jun, and the basal transcriptional apparatus (e.g. TFIIB) that may be required for full transcriptional activation. Furthermore, RNA polymerase II constitutively associates with CBP/p300 and, as such, interaction with p65 may recruit the polymerase to the gene promoter. The interaction of CBP/p300 with CREB is dependent upon phosphorylation of CREB by PKA, and therefore the interaction of CBP with p65 may also be modulated by PKA. Interestingly, the three-dimensional structure of p65 indicates that ser-276 is in a loop in the dimerization domain, which is exposed to the surface (39). Hence phosphorylated p65 can provide an excellent binding surface for coactivators such as CBP/p300. However, evidence demonstrating that PKA phosphorylation modulates the interaction between CBP and p65 is yet to be provided.

KNOCKOUTS OF NF-κB AND IκB PROTEINS: WHAT DO THEY TELL US ABOUT THEIR PHYSIOLOGICAL ROLES?

Knockouts of NF-κB/Rel Proteins

The presence of different rel family members in most cell types poses a challenge in understanding the distinct biological role of each family member. Therefore, to further delineate the exact role of each rel protein in vivo, targeted gene disruption in mice has been performed. Several groups have independently knocked out many of the rel proteins including p50, p52, c-rel, p65 (Rel A), and Rel B (146–151).

Because p50 is a shared component of most NF-κB complexes that regulate genes involved in immune function and immune cell development, it was anticipated that a p50 knockout would be lethal. Remarkably, the absence of p50 does not cause developmental abnormalities or lethality by itself. These mice have normal levels of B cells and normal ratios of κ and λ light chain

usage (150). However, B cells isolated from these mice cannot be induced to differentiate in response to the mitogen LPS, and no NF-κB activation was observed. In contrast, cross-linking IgM with appropriate antibodies does induce NF-κB (p65 and c-Rel, but not p50) and B cell proliferation (150). This result strongly suggests that these two stimuli use distinct signaling pathways. Interestingly, p50 also seems to play a crucial role in isotype switching since the levels of serum IgA, IgE, and IgG1 in knockout mice were significantly lower than in control littermates. Not surprisingly, these mice had dramatically greater susceptibility to bacterial infection but remarkably enhanced resistance to viral infection. This latter effect was due to an upregulation of the antiviral cytokine IFN-β. NF-κB is thought to upregulate the levels of this protein, but this result indicates that p50 mediates a repressive effect in this particular system, probably acting as p50 homodimers. Therefore, it appears that p50 plays a very important role in immune responses to acute inflammation but not in the overall development of the mouse. It remains possible, however, that since p50 is a member of a larger family of proteins, another rel protein may be compensating for its loss in the p50 knockout mice.

Expression of c-rel is generally restricted to hemopoietic cells (152). Specifically, it is found in the greatest amount in T and B cells and appears to be a major component of NF-κB found in the nucleus of B cells. Nevertheless, the absence of c-rel, like that of p50, has few consequences on the development of hemopoietic cells (149). The number of these cells appears to be equivalent to that of normal littermates, and cell surface markers such as Igκ and the IL-2Rα chain are expressed at normal levels. C-Rel appears instead to have an effect on the inducible response of B cells and T cells to mitogen stimulation (149). Thus, the proliferative and humoral response of both B and T cells is reduced in c-rel$^{-/-}$ mice as is the level of IL-2. Interestingly, the composition of nuclear NF-κB dimers from B cells differs in c-rel$^{-/-}$ mice relative to normal mice. More specifically, the absence of c-rel appears to be compensated by the presence of more p65 in the nucleus. This compensation however is obviously not functional because c-rel-deficient mice have profound lymphocyte activation defects.

In contrast to p50, p65 has a critical role in development: Mice lacking this gene die at day 16 of gestation (146, 148). The livers from the resulting embryos undergo massive degeneration as a result of apoptosis. It is unclear whether the absence of p65 mediates apoptosis of hepatocytes directly, or whether p65 regulates expression of an additional factor that is necessary for survival. Obviously, there is no functional complementation effect here, suggesting that this particular developmental parameter is specific to p65.

Two groups have inactivated the RelB gene (147, 151). Unlike p50 and p65, which are expressed in virtually all cell types, RelB expression is largely

restricted to lymphoid tissue. Interestingly, the effects of this knockout are biphasic, since the mice develop normally until about day 10 after birth. However, after this time the mice begin to express a severe and complex phenotype including thymic atrophy, splenomegaly, hyperplasia in the bone marrow, and a dysfunctional immune response; in extreme cases this leads to premature death (151). Burkly and colleagues have further suggested that Rel B plays a critical role in development of dendritic cells in the bone marrow and UEA-1+ medullary epithelial cells in the thymus; they conclude that RelB could be a candidate gene for committing precursor cells to a particular lineage within the immune system (147). RelB appears to have little effect on lymphoid development, although there is evidence to suggest that it may be important in T cell–mediated immunity. Interestingly the multiorgan inflammation and myeloid hyperplasia observed in RelB-deficient mice are dependent on T cells, since crossing the knockout mice with Nur 77/N10 transgenic mice lacking T cells blocks the development of disease symptoms (153).

The phenotypes of these knockout mice lacking individual Rel-proteins demonstrate the importance of proteins in this family in different cellular processes. It is clear that the different members serve unique functions that cannot be fully complemented by other Rel-proteins. However, the possibility remains that complementation by family members masked some phenotypes and hence prevented the complete elucidation of the physiological roles of the Rel-transcription factors. Recent studies have attempted to address such possibilities by generating knockouts of multiple Rel-family members. The first study examined the effect of generating p50/Rel B double knockout mice (154). The inflammatory phenotype observed in the relB$^{-/-}$ mice was greatly exacerbated in the double knockout mice and led to death of the mice within four weeks. Therefore, it appeared that the lack of RelB is in fact compensated by other p50 containing complexes, but the inflammatory phenotype is not due to the p50:p65 NF-κB. The second double knockout removed both p50 and p65 (155). The knockout of p65 alone resulted in an embryonic lethal phenotype that prevented examination of the possible role of p65 in lymphocyte development. To overcome this difficulty, two groups transplanted fetal liver stem cells from p65$^{-/-}$ mice into either lethally irradiated or SCID mice (148, 155). Surprisingly stem cells lacking p65 developed normally and reconstituted hematopoetic lineages in a manner similar to that seen when fetal liver cells from wild-type or heterozygous animals were used. To determine whether deletion of p50 along with p65 might reveal a greater effect, Horwitz et al generated p50$^{-/-}$, p65$^{-/-}$ mice through crosses (155). These double knockout mice were also embryonic lethals with massive apoptosis of liver cells. However, the onset of apoptosis was at embryonic day 12 instead of day 16 in case of p65$^{-/-}$ mice. When

fetal liver cells from day 12 of these mice were used to complement lethally irradiated animals, there was no observable development of lymphocytes (155). This dramatic block in lymphocyte development of the p50/p65 double knockout cells could be rescued if bone marrow cells from wild-type animals were co-injected into the recipient animals. The only logical conclusion from this result is that NF-κB plays a critical role in lymphocyte development. However, this role is manifested not in the developing lymphocytes but in surrounding cells. These surrounding cells probably produce a critical growth factor whose expression is dependent on NF-κB. These results are highly intriguing, and further study will be necessary to elucidate the relationship between NF-κB and lymphocyte development.

In summary, the results of the different knockouts reveal that individual members of the Rel-family play distinct and unique roles that cannot be replaced by other members of the Rel-family. Thus, p50 and c-rel appear to be more important in mediating acute immune responses than in overall development. p65 and RelB, on the other hand, have crucial roles in the development of the liver and the haematopoietic system, respectively, and their absence may not be compensated for by other rel family members. Enhancement of the observed phenotypes in the double knockouts (154, 155) suggests that there is some functional complementation, and the degree to which this occurs may differ among the different family members as well as in different tissue or cell types. Efforts are underway to generate more knockouts, and it is likely that through this approach we will have a far better understanding of the physiological role of Rel-proteins in the near future.

Knockouts of IκB Proteins; IκBα and Bcl-3

Recently mice lacking the IκBα gene have been generated, and although the pups are born normally (i.e. there are no developmental defects), they die within 9 days of birth (156, 157). Typically, the animals show rapid physiological degeneration including atrophy of the spleen and thymus, severe runting, and the development of many skin abnormalities (which may result from enhanced granulopoiesis). In addition the levels of constitutive NF-κB activity are greatly enhanced in haematopoietic organs (156). It remains unclear whether the enhancement of NF-κB activity in lymphoid tissue is the result of the loss of anchoring function of IκBα or whether it is due to the inability of IκB$\alpha^{-/-}$ mice to terminate NF-κB activation in these cells. To determine whether the enhanced NF-κB activity is responsible for this lethal phenotype, a chimeric IκB$\alpha^{-/-}$p50$^{-/-}$ mouse has been generated (156). Here, constitutive NF-κB levels are lower than in the IκB$\alpha^{-/-}$ mouse, and the onset of the lethal phenotype is delayed until the third week after birth. The absence of p50 therefore

suppresses but does not eliminate the effects of NF-κB overexpression and suggests that constitutive activation of the Rel proteins is a primary cause of neonatal lethality in the IκB$\alpha^{-/-}$ mouse.

These results also begin to illuminate the interplay between IκBα and IκBβ in regulating NF-κB expression (156, 157). The most obvious conclusion from these studies is that IκBβ is incapable of inhibiting the constitutive expression of NF-κB and thus cannot substitute for IκBα; this suggests that there are IκBα- and IκBβ-sensitive pools of NF-κB within cells that may be targeted by different signal transduction pathways. Furthermore in embryonic fibroblasts, constitutive NF-κB activity is low, but in the presence of TNF-α, significant NF-κB is activated (156, 157). Suppression of this inducible activation appears to require IκBα because the levels of NF-κB remain high in these cells after removal of the activating signal. By contrast, in similar experiments in wild-type cells, the levels of NF-κB rapidly returned to the basal level due to the sequestering of NF-κB from the nucleus by IκBα. The source for NF-κB in these TNF-stimulated fibroblasts appears somewhat controversial; Beg et al (156) suggest that it is due to the degradation of IκBβ, whereas Klement et al (157) do not observe any changes in IκBβ levels. More recently it has been proposed that IκBβ levels are highly upregulated in IκB$\alpha^{-/-}$ mice and that the source of induced NF-κB in TNF-stimulated fibroblasts may be cytoplasmic IκBϵ complexes (69). It is however fair to conclude that the phenotype of the IκB$\alpha^{-/-}$ mice supports the data from cell lines, which suggests that IκBα is more important in regulating inducible responses, whereas IκBβ is involved in the persistent activation of NF-κB. Generation of mice lacking IκBβ will be necessary to conclusively test the validity of this model.

Two groups have recently reported the generation of mice lacking the *bcl-3* gene (158, 159). The paradoxical nature of Bcl-3, which resembles IκB proteins but activates NF-κB activity, and its low level of expression had prevented meaningful analysis of its in vivo function. In addition, expression of *bcl-3* in thymocytes of transgenic mice indicated that instead of blocking, overexpressed Bcl-3 augmented DNA binding by p50 dimers (160). The knockout mice did not reveal any differences in the expression of NF-κB and IκB proteins, induction of NF-κB activity, or composition of the NF-κB complexes detected in these animals. No defects in development appeared either in the animals or in the immune system. Instead they display selective defects in the response to immunogenic challenge from pathogens such as *L. monocytogenes, S. pneumoniae*, or *T. gondii.* Some of these defects could be due to the fact that these mice lack germinal centers and display altered microarchitecture in the spleen and lymph nodes. None of the observed phenotypes shed any light on the basis of the oncogenic potential of *bcl-3*, although the somewhat reduced numbers of B cells in these mice may reflect a role of *bcl-3* in the survival of B cells.

Interestingly, mice lacking the p52 protein, the presumed partner for nuclear complexes with Bcl-3, display defects similar to that of Bcl-3, namely, lack of germinal centers and inability to respond to certain pathogens (161, 162).

Currently only one cytosolic anchor of NF-κB activity, namely IκBα, has been knocked out. Whereas lack of certain Rel-members (p65) leads to lethality, paradoxically, the targeted deletion of IκBα also promotes neonatal lethality as a result of an overexpression of the Rel proteins. It is therefore evident that precise regulation of NF-κB expression both temporally and spatially is necessary to ensure normal development and subsequently to achieve an effective immune response against antigenic challenge.

NF-κB and Apoptosis

The role of Rel-proteins in oncogenic transformation is quite well established (see Gilmore & White 1996 for a recent comprehensive review on this area) (163). Transforming forms of Rel-proteins include v-Rel, lyt-10, and truncated forms of c-Rel. The mechanism underlying Rel-mediated transformation remains unclear despite years of study, particularly because evidence connecting Rel-function with growth regulatory mechanisms has not been forthcoming. The phenotype of the $p65^{-/-}$ mice, which die during embryonic development through massive apoptosis of hepatocytes, suggested that instead of promoting growth of cells, NF-κB might play an important role in protecting cells from apoptosis (146, 148). Hence the transforming effect of Rel-proteins may be the consequence of their anti-apoptotic function, not unlike the tumors induced by oncogene Bcl-2.

Strong support for a protective role for NF-κB in apoptosis came from four recent reports that examined the effects of TNF on cell killing (115, 164–166). The cytokine TNF was initially characterized by its ability to kill tumor cells. However subsequent analysis indicated that its ability to kill cells was highly cell-type dependent. The recent reports indicate that inhibiting NF-κB activity in cells, either in cell lines lacking p65 or those expressing a dominant negative form of IκBα, made cells that were otherwise insensitive to TNF-mediated killing exquisitely susceptible to apoptosis. Interestingly, this protective function of NF-κB was apparent not only against TNF but also on cells treated with ionizing radiation or chemotherapeutic agents, thus suggesting that NF-κB may play an anti-apoptotic role in many systems (166). There is also evidence that inhibition of the constitutive NF-κB activity in WEHI 231 cells prevents their apoptosis (167). Because in most cases the enhancement of apoptosis can also be manifested by blocking protein synthesis using cycloheximide, it appears that NF-κB probably serves to upregulate the synthesis of one or many anti-apoptotic gene products, e.g A20 (168). Identification of such NF-κB regulated genes therefore is an important objective for the near future.

As discussed above, the phenotype of the double knockouts indicates some functional complementation between members of the Rel-family, thus keeping alive the possibility that the only way to generate a mouse lacking all NF-κB activity would be to create one with all the members missing. Although this might soon be possible, a few reports present an alternate strategy. In these papers, a dominant negative form of IκBα, lacking the N-terminal signal-responsive domain, is expressed in a tissue-specific fashion to inactivate all NF-κB activity. Studies carried out in cell lines clearly establish the validity of this approach (165, 166, 169), and a recent report extends such studies to examine the regulation of NF-κB in T lymphocytes in transgenic mice (170). The dominant negative IκBα was expressed in thymocytes using a 3′ lck promoter (with co-integrated CD2 promoter) and led to a surprising result. The ratio of single positive T cells was altered in these mice with a significant loss of CD8 cells. The reason behind this alteration in the profile of single-positive T cells remains unclear; however, the loss of CD8 cells without a corresponding increase in CD4 cells suggests that defect is not at the stage of commitment to the CD4 or CD8 lineages. Instead the observed results probably derive from a selective apoptosis of CD8 cells, an explanation that would reinforce the possible role of NF-κB as an anti-apoptotic gene. Further use of this approach should help uncover other systems where NF-κB might play similar roles.

SUMMARY

The study of the regulation of transcription factors of the Rel family appears poised to achieve significant breakthroughs in the near future. Particularly interesting will be the identification and characterization of the putative IκB kinase. Events that follow the signal-induced phosphorylation of IκBs, namely the ubiquitination and degradation of the protein, also appear to be an important area of future study. Finally, the role of NF-κB in modulating the expression of so many different cytokines and lymphokines strongly supports its proposed role as a co-ordinating element in the body's response to situations of stress, infection, or inflammation. Therefore, development of specific inhibitors of its function should lead to novel therapeutics, which may serve to effectively treat situations such as inflammation.

ACKNOWLEDGMENTS

We would like to acknowledge the help of Dr. Dola Sengupta and Dr. Roderick Phillips in the preparation of this manuscript. The authors' own research was supported by grants from National Institutes of Health and the Howard Hughes Medical Institute.

NOTE ADDED IN PROOF Following submission of this review, two groups reported the identification and characterization of the IKKα kinase that displays many of the expected properties for a bona fide IκB kinase (172, 173). The sequence of the cDNA encoding this kinase revealed that it was the same as a previously cloned kinase (CHUK) of unknown physiological function. The kinase NIK, which appears to be a component in the pathways originating from inducers such as TNF-α and IL-1, was found to associate with IKKα, but the significance of this association is not completely clear. Interestingly, IKKα appears to be a phosphoprotein, and treatment with protein phosphatase 2a (PP2a) leads to a loss of activity, a result in agreement with the capacity of okadaic acid to activate NF-κB in intact cells. The purified kinase complex, which includes IKKα, is ≈900 kDa in size, and hence the characterization of other components of the kinase complex is an important question that remains to be solved.

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Annual Review of Immunology
Volume 16, 1998

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0022-1767/91/1475-1694802.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 147, 1694–1700, No. 5, September 1, 1991
Printed in U.S.A.

ENHANCERS AND TRANSCRIPTION FACTORS CONTROLLING THE INDUCIBILITY OF THE TUMOR NECROSIS FACTOR-α PROMOTER IN PRIMARY MACROPHAGES

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In macrophages, the TNF-α promoter is specifically induced by bacterial endotoxin, and provides a good model for gene regulation during bacterial infections. We have analyzed the protein-binding characteristics and enhancer activity of four κB-like enhancers and of a MHC class II-like Y box found in the mouse TNF-α promoter. In addition to members of the NF-κB/rel transcription factor family, at least two of the κB sites also bound a nuclear protein identified as NF-GMa, a factor that binds to promoter sequences from many cytokines. When inserted upstream of an enhancer-less promoter, two of the κB sites were active as LPS-inducible enhancers in primary macrophages, whereas the other two were not. Mutations in nucleotides known to contact nuclear factors severely reduced affinity of the κB sites for NF-κB. Introduction of the same mutations into a construct containing 1059 bp of the TNF-α promoter coupled to a CAT reporter gene resulted in a stepwise reduction in inducibility by LPS; mutation of all four sites (11 bp of 1059) reduced inducibility by 90%, providing compelling evidence for the role of transcription factors belonging to the NF-κB/*rel* family in the activation of the TNF-α promoter. The TNF-α Y box bound an abundant nuclear factor, but had no detectable activity in our assays, either as an enhancer or as a mutation-sensitive controlling element.

Macrophages play a key role in the initiation of a normal inflammatory response through their ability to synthesize multiple cytokines upon exposure to bacterial products, in particular the endotoxin or LPS component of Gram-negative cell walls (1). The genes coding for many of these cytokines have been characterized over the last few years; however, surprisingly little is known about the molecular mechanisms of LPS-mediated gene induction in macrophages, particularly at the transcriptional level.

The synthesis of TNF-α is very strongly and specifically induced by LPS, and overproduction of TNF-α by macrophages is thought to be a major cause of endotoxic shock (2). Increased transcription accounts for a significant portion of the induction of TNF-α production, although posttranscriptional events also play a major role (3–6). When mouse TNF-α promoter/CAT hybrid constructs are transfected into primary macrophages, sequences extending more than 450 bp upstream of the mRNA initiation site are necessary for full inducibility by LPS (7). Loss of inducibility in a 5′ deletion series correlates with the deletion of sites capable of binding the NF-κB transcription factor, and one of the κB binding sites is able to function as an LPS-inducible enhancer. Moreover, LPS is a very potent inducer of NF-κB in macrophages (7–9). These results strongly suggested a role for NF-κB in the LPS-mediated induction of TNF-α transcription. However, a recent study of the regulation of the human TNF-α promoter in transformed cell lines found no evidence for an LPS-inducible enhancer activity of its NF-κB binding sites (10).

Recently, a motif related to but distinct from the κB enhancer was reported to be involved in the LPS inducibility of the gene coding for G-CSF, another cytokine produced by activated macrophages (11). This motif, which was called cytokine-1 or GPE-1, acts as a TNF-inducible enhancer in embryonic fibroblasts (12), and is found in the promoters of many cytokines (13). Although its sequence bears a strong resemblance to enhancers of the κB family, it binds a protein (NF-GMa) distinct from NF-κB (12). The κB sites that we identified in the TNF-α promoter also fit the consensus for the CK-1 motif. Therefore, NF-GMa may contribute to the LPS inducibility of the TNF-α promoter.

Our previous results suggested that a sequence identical to the Y box of MHC class II promoters may play a role in the regulation of TNF-α transcription (7). The Y box is a member of the "CCAAT box" family of upstream regulatory elements, and binds one of the CCAAT-binding factors, CP-1/NF-Y (14). The Y box is thought to play a role in the baseline expression of MHC class II promoters rather than in their inducibility.

The experiments described in this paper attempt to assess the relative contributions of DNA sequences binding NF-κB, NF-GMa, and NF-Y to the LPS inducibility of the TNF-α promoter.

MATERIALS AND METHODS

Artificial enhancer constructs. Double-stranded oligonucleotides containing 5′ overhangs compatible with restriction endonuclease-generated ends (Fig. 2) were introduced into pBLCAT2, a vector that

Received for publication February 11, 1991.
Accepted for publication June 11, 1991.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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contains 105 bp of the HSV TK promoter linked to the CAT reporter gene (15). The κB1 and κB4 oligonucleotides were introduced into the *Bam*HI site, κB3 into *Hin*dIII, κB2 into *Xba*I (after filling in with DNA polymerase), and the Y box into *Sal*I. The copy number and orientation of the inserts was verified by sequencing. The plasmid containing the Y box upstream of the TNF-β promoter was constructed by inserting a *Pvu*II to *Bam*H I fragment (nt −287 to +81) of TNF-β into pBLCAT2 containing one copy of the Y box (in the *Sal*I site), cut with *Xba*I (blunt-ended) and *Bam*HI. The *Bam*HI to *Bgl*II fragment containing the HSV TK promoter was subsequently deleted from this construct.

Site-directed mutagenesis of the TNF-α promoter. The TNF promoter constructs were derived from a λEMBL3 genomic clone containing most of the mouse (strain C57Bl/6) TNF locus (16). To generate the mutations in sites κB2, κB3, κB4, and the Y box, oligonucleotide-directed in vitro mutagenesis was performed according to Taylor et al. (17), using a kit from Amersham Corp. (Amersham, UK). The mutations introduced are indicated in Figure 2; mutagenic primers were annealed to ssDNA template prepared from a phage carrying the *Bam*HI-*Nar*I (−1059 to +138) fragment of the TNF-α promoter inserted at the *Bam*HI and *Acc*I sites of the mWB2311 vector. mWB2311 is identical to mWB238 (18), but contains the polylinker from M13mp11. The mutation in κB1 was generated by PCR using mismatched primers (19). After subcloning into pBLCAT3, the sequence of the entire mutated inserts was verified using the dideoxy nucleotide chain termination protocol.

Electrophoretic mobility shift assays. Total cell extracts were prepared according to Schreiber et al. (20) from bone marrow-derived macrophages or from EL4 cells; activation by LPS (100 ng/ml) or by PMA (5 ng/ml) was for 3 h. Restriction fragments or oligonucleotides were labeled with the Klenow fragment of DNA polymerase I or with T4 polynucleotide kinase. The SV40 κB enhancer probe was isolated from SPT-TCII (21), a plasmid containing four tandem copies of nt 233-247 (BBB numbering system) from the SV40 genome. For binding reactions, 0.1 to 10 ng of end labeled fragment (5,000–20,000 cpm) was mixed with 16 μg (protein) of nuclear extract in a total volume of 10 μl containing 25 mM HEPES pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 12% (v/v) glycerol, 40 mM KCl, 100 μg/ml poly(dI:dC), and 25 μg/ml denatured *Escherichia coli* DNA. After 30 min of incubation at room temperature, the reaction mixtures were loaded on 4% polyacrylamide gels in low ionic strength buffer (0.25 × TBE: 22 mM Tris-borate, 0.5 mM EDTA). The gels were prerun for at least 1 h at 20 V/cm and run for 3 h at 18 V/cm at 4°C. After electrophoresis, the gels were dried and exposed for autoradiography.

Transfection of bone marrow-derived macrophages. Macrophages were generated from bone marrow cells of C57Bl/6 mice and transfected using DEAE-dextran as previously described (7). LPS (form W from *E. coli* O55:B5; Difco Laboratories, Detroit, MI) was added 24 h after transfection. CAT assays were performed on crude extracts 36 h after transfection, using ^{14}C-labeled chloramphenicol (Amersham plc, Amersham, UK) as a substrate, and TLC to separate the native and acetylated forms.

RESULTS

Proteins binding to TNF-α promoter upstream sequences. The sequences within 1 kb upstream of the mouse TNF-α mRNA initiation site contain a perfect MHC class II Y box at position −255 and at least four sites with significant similarity to the κB enhancer, located around positions −850 (κB1), −655 (κB2), −510 (κB3), and −210 (κB4) (Figs. 1 and 2). All 4 of these sites have been shown to bind NF-κB, although binding to site κB4 was reported to be very weak (8). In accordance with the results of Collart et al. (Fig. 6 in Ref. 8) we found that in addition to NF-κB, sites κB2 (strongly) and κB1 (weakly) bound a constitutively expressed factor, producing a slower-migrating band that we called band C, and that site κB1 produced an additional specific band that we called D. The inducible and constitutive forms of NF-κB (8) were termed bands A and B, respectively. Our binding and competition assays established a hierarchy of binding affinities between sequence motifs and nuclear factors: κB3 > κB1 > κB2 > κB4 for bands A and B; κB2 > κB1 > κB4 ≫ κB3 for band C; κB1 ≫ κB2 ≅ κB3 ≅ κB4 for band D.

To further characterize the DNA-binding factor(s) responsible for the formation of band C, we performed the competition assay depicted in Figure 3, using the κB2 oligonucleotide as a probe. An oligonucleotide derived from the CK-1 site of the G-CSF promoter, the canonical binding site for NF-GMa (12, 13) strongly competed for the formation of band C, whereas the κB3 oligonucleotide, which has a high affinity for NF-κB, competed very weakly. Conversely, labeled G-CSF oligonucleotide produced band C, which could be efficiently competed with cold kB2 (data not shown). The Y box oligonucleotide did not have any inhibitory effects in these assays (not shown).

Enhancer activity of TNF promoter sequences. To test the LPS-inducible enhancer activity of the DNA sequences described above, we introduced them in various combinations and copy numbers upstream of the enhancer-less HSV TK promoter of the pBLCAT2 vector (15). The resulting plasmids were transfected into bone marrow-derived macrophages, which were then tested for LPS-inducible expression of the CAT gene (Table I). As described earlier (7), site κB3 had an LPS-inducible enhancer activity that was expressed maximally when it was present in three or more copies. Two or more copies of site κB2 also conferred LPS inducibility upon the HSV TK promoter. The enhancer activity of site κB2 was confirmed by testing a plasmid containing one copy each of sites κB2 and κB3. Although one copy of κB2 or κB3 was not sufficient to produce an inducible phenotype, the combination of both acted synergistically, and the resulting plasmid was more inducible than those carrying two copies of either site.

The two other NF-κB binding sites were tested in a fashion similar to κB2, either alone in multiple copies or in combination with one copy of κB3 (Table I). No enhancer activity could be detected for κB1 or κB4 in these assays, even when the sites were present in multiple copies and/or adjacent to a copy of κB3. This was particularly unexpected for κB1, which binds NF-κB with high affinity.

The Y box was similarly tested, and found not to have any LPS-inducible enhancer activity, either alone or in combinations with κB2 or κB3 (Table I). To test whether the Y box could allow expression in macrophages of an otherwise silent promoter, we constructed a plasmid containing one copy of the Y box 290 nt upstream of the mRNA initiation site of the mouse TNF-β promoter, which is not expressed in macrophages, even though it contains a perfect κB consensus. Addition of this Y box was ineffective (data not shown).

Effects of point mutations on protein binding. To further test the importance of the NF-κB binding sites

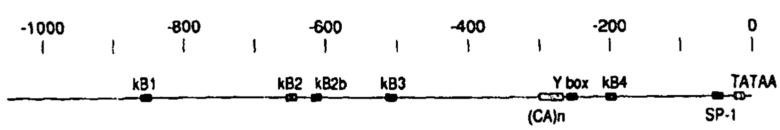

Figure 1. Structure of the mouse TNF-α promoter. The sequence elements discussed in this paper and their location relative to the mRNA start site are indicated. The sequences of the TATAA box and Sp-1 site can be found in Shakhov et al. (7).

kB site 1 (-850)
C T T G
5'G A T C T G G G A G G G G A A T C C T T G G A A
A C C C T C C C C T T A G G A A C C T T C T A G 5'
G A A C

kB site 2 (-655)
A A
5'T T G T G A G G T C C G T G A A T T C C C A G G G C T G A G T T C A
A C A C T C C A G G C A C T T A A G G G T C C C G A C T C A A G T A 5'
T T

kB site 3 (-510)
A A
5'A G C T C A A A C A G G G G G C T T T C C C T C C T C
G T T T G T C C C C C G A A A G G G A G G A G T C G A 5'
T T

kB site 4 (-210)
A T C
5'G A T C C G G A G G A G A T T C C T T G A T G G
G C C T C C T C T A A G G A A C T A C C C T A G 5'
T A G

Y box (-255)
A T T C
5'T C G A C C C T C C T G A T T G G C C C C A G A T
G G G A G G A C T A A C C G G G G T C T A A G C T 5'
T A A G

Figure 2. Oligonucleotides used in this study. The Figure shows the sequences of both strands of the four κB oligonucleotides and of the Y-box oligonucleotide, including the overhangs used for cloning. The same oligonucleotides were used in the EMSA. The *shaded regions* indicate nucleotide changes in the mutants.

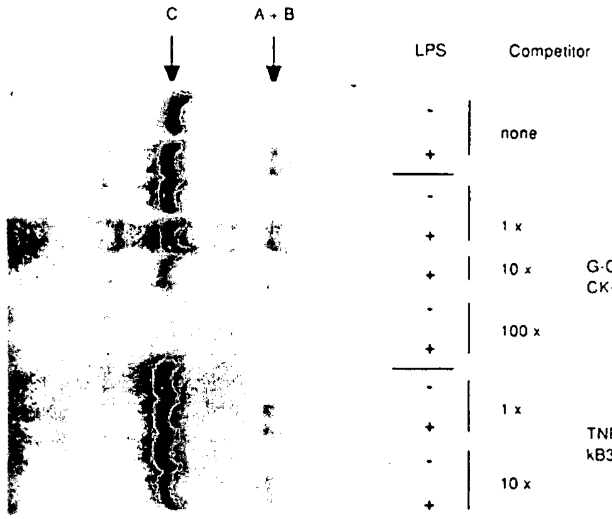

Figure 3. Competition with the G-CSF CK-1 site. A labeled oligonucleotide containing the κB2 site was mixed with nuclear extracts from resting (−) or LPS-activated (+) macrophages. The indicated amounts of cold oligonucleotides were added as competitors. The sequence of the G-CSF oligonucleotide was 5' CTAGAGGAGGATCA**GAGATTCCACAATTTCACAT** 3' (consensus CK-1 site in boldface).

TABLE I
Enhancer activity of protein-binding DNA sequences from the TNF promoter

DNA Sequence (No. of Copies)					LPS-Induced CAT Activity[a] (Percent of wt)
κB 1 (-850)	κB 2 (-655)	κB 3 (-510)	κB 4 (-210)	Y box (-465)	
					<5
1-5					<5
1-3		1			<5
	1				<5
	2-3				18 ± 3.5
	1	1			30 ± 10
		1			<5
		2			9 ± 2.5
		3			90 ± 22
			1-3		<5
		1	1-3		<5
				1-3	<5
		1		2-5	<5
	1			1-4	<5

[a] pBLCAT2-derived plasmids containing TNF-α promoter upstream elements in the indicated combinations were transfected into primary macrophages and assayed for inducibility by LPS. The values shown were derived from at least three experiments using at least two different DNA preparations, and were normalized to the activity obtained with a wild-type promoter in the same experiment. CAT activity in the absence of LPS was undetectable in all cases.

and the Y box for the LPS-inducible expression of the TNF promoter, we synthesized oligonucleotides mutated at positions thought to be important for the formation of specific DNA-protein complexes (Fig. 2). In particular, we modified guanosines known from methylation interference assays to make contact with protein (8). We tested the affinity of the mutated oligonucleotides for NF-κB in a competition assay, using a tetramer of the SV40 κB enhancer as a probe. The mutations reduced the ability of the oligonucleotides to compete with the SV40 enhancer by more than 10-fold; for all four κB oligonucleotides that we tested, a 200-fold excess of the mutant displaced no more than 50% of the probe, whereas an equivalent amount of wild-type oligonucleotide abolished binding completely. As the Y box oligonucleotide (irrelevant competitor) did not compete measurably in this assay, the mutated κB sites probably still have some residual NF-κB binding activity.

We then used site-directed mutagenesis to introduce the same base substitutions into TNF(−1059)CAT, a construct containing more than 1 kb of the mouse TNF promoter linked to a CAT reporter gene (7). We assayed the effects of the mutations on the formation of DNA-protein complexes with larger regions of the promoter, using restriction fragments as probes (Fig. 4). A fragment spanning nt −695 to −432 formed two major protein-DNA complexes, one of which was inducible (complex I) and the other constitutive (complex II). Complex II was not formed with a fragment mutated at the κB2 site, suggesting that it contains the constitutive factor(s) that form band C with the κB2 oligonucleotide. Mutation of κB2 affected formation of complex I only marginally. However, mutation of both sites prevented the formation of both complexes, as did mutation of κB3 alone. The latter result could be explained if NF-κB binding to κB3 stabilized the complex of NF-GMa with site κB2: only complexes with low dissociation constants can be detected in mobility shift assays using large DNA fragments as probes. Mutation of the Y box did eliminate the appearance of the Y box-specific LPS-inducible band (Fig.

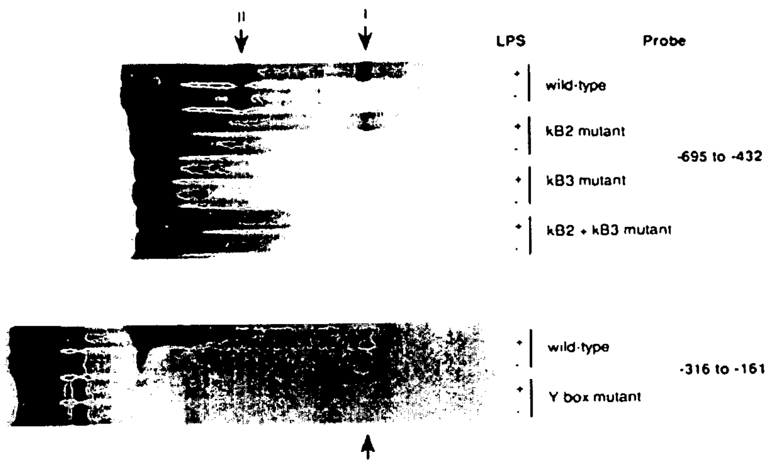

Figure 4. Effect of mutations on complex formation by restriction fragments. Restriction fragments spanning nt −695 to −432 (*SacI* to *NcoI*) or −316 to −161 (*AvaII* to *FnuDII*) were prepared from wild-type or mutant TNF-α promoters and subjected to EMSA using extracts from resting or LPS-activated macrophages.

4, *arrow*) (7) when we used a probe spanning nt −316 to −161.

Effects of mutations on LPS inducibility of the TNF promoter. We tested the mutated forms of the TNF promoter for inducibility by LPS in our macrophage transfection assay (Table II). While individual mutations of the κB sites reduced inducibility by no more than 50%, the inactivation of additional sites gradually diminished the response of the promoter to LPS. In a construct where all four of the sites identified above had been inactivated, the response to LPS was reduced by about 90%. The degree of inactivation (Table II) did not correlate with the ability of individual sites to act as LPS-inducible enhancers (Table I). In particular, although mutations of κB2 and κB3 (both of which acted as LPS-inducible enhancers) resulted in about 50% inactivation, additional mutations of κB1 or κB4 (which had no enhancer activity by themselves) each brought about a further twofold reduction in inducibility. The combined effects of the mutations were approximately multiplicative, with each additional mutation reducing inducibility by about 50%.

We tested the effects of mutating the Y box, with consistently negative results (Table II). Promoters with a mutated Y box, combined or not with mutations in the NF-κB binding sites, were at least as inducible as the equivalent constructs containing a wild-type Y box. We also made a 4 bp deletion in the Y box, with similar results (data not shown).

TABLE II

Effects of mutations in the TNF promoter on inducibility by LPS

Mutations in Binding Sites					LPS-Induced CAT Activity[a] (Percent of wt)
κB 1 (-850)	κB 2 (-655)	κB 3 (-510)	κB 4 (-210)	Y box (-465)	
mut					59 ± 3
	mut				58 ± 15
		mut			54 ± 16
			mut		48 ± 5
				mut	104 ± 15
	mut	mut			47 ± 13
	mut		mut		40 ± 11
		mut	mut		36 ± 13
mut	mut	mut			19.5[b]
	mut	mut	mut		27 ± 8
mut	mut	mut	mut		10 ± 2
	mut			mut	52 ± 18
		mut		mut	68 ± 16
	mut	mut		mut	67 ± 30

[a] Plasmids containing mutated forms of the TNF-α promoter (see Fig. 1) were transfected into primary macrophages and assayed for inducibility by LPS. The data are presented as in Table I.

[b] Only one experiment available for this mutant. All the other constructs tested in the same experiment behaved as expected.

DISCUSSION

The experiments described in this report attempted to estimate the relative contributions to the LPS inducibility of the TNF-α promoter of a set of DNA sequences that bind nuclear proteins. The results may be best discussed in terms of the transcription factors that these sequences bind.

NF-κB. There is now ample evidence that LPS induces the appearance of NF-κB in the nucleus of macrophages (7–9), and four of the sites that we selected for study have been shown to bind NF-κB (8). It was unexpected that the LPS-inducible enhancer activity of the κB sites did not correlate with their affinity for NF-κB. Site κB1, which has the second highest affinity for NF-κB, was totally inactive as an enhancer (Table I). The inducible enhancer activity of site κB2 may be due to both its ability to bind NF-κB (albeit with a low affinity) and its high affinity for the factor(s) present in complex C, which is almost certainly NF-GMa (see below). By the criteria of NF-κB binding and enhancer activity, site κB4 could be dismissed as irrelevant to the process of LPS induction. However, the data obtained with mutant promoters give a different picture: mutations in each of the κB sites reduced inducibility by LPS, irrespective of their affinity for the factor or their enhancer activity (Table II). The simplest explanation for this discrepancy is that there may be cooperative effects, and that the occupancy of one site could influence the binding of factors at other sites. Some support for this interpretation comes from the fact that a mutation at site κB3 (which only binds NF-κB) prevented the detection of a constitutive complex that was also affected by a mutation at site κB2 (complex II in Fig. 4). A fifth site in the TNF-α promoter also resembles a kB enhancer (Fig. 5), and has been shown to bind NF-κB (S. A. Nedospasov, personal communication). This site may be responsible for the residual inducibility by LPS of our quadruple mutant (Table II). The data presented here and elsewhere (7, 8) provide compelling evidence for a central role of transcription factors related to NF-κB in the LPS-mediated induction of the mouse TNF-α promoter.

A recent report by Goldfeld et al. (10), which came to our attention after the experiments described here were

		Sequence	Binds NF-κB	Binds NF-GMa	LPS-inducible enhancer
kB consensus (Lenardo & Baltimore, 1989)		GGGRYTYYCC			
SV40, Ig kappa: kB paradigm		GGGGACTTTCC	+++	-	+++
GM-CSF1 (mouse)		GGTAGTTCCCC	+++	?	?
TNF-alpha (mouse)	3 (-510)	GGGGGCTTTCC	+++	-	+++
	2b (-610)	TGGGGCTGCCC	++	?	?
	1 (-850)	GGGGAATCCTT	++	+	-
	2 (-655)	CGTGAATTCCC	+	+++	++
	4 (-210)	GGAGATTCCTT	+	++	-
G-CSF (human)		AGAGATTCCAC	?	+++	? (TNF: ++)
GM-CSF2: CK-1 paradigm		GGAGATTCCAC	+	+++	?
CK-1 consensus (Shannon et al., 1990)		GRGRTTYCYN			

Figure 5. Comparison of κB and CK-1 motifs from cytokine promoters. The sequences, protein-binding properties, and LPS-induced enhancer activity of κB-like elements from different promoters are compared. Purines are in boldface and pyrimidines in italics, and the most conserved positions are shaded. The data on protein binding and enhancer activity are from this report, and from Shannon et al. (12, 13), Schreck and Baeuerle (24), and the review by Lenardo and Baltimore (32).

completed, provides evidence against a role for NF-κB in the inducibility of the human TNF promoter by LPS or by viral infection. Previous studies of the inducibility of the human promoter by PMA (22, 23) also failed to indicate a role for NF-κB. One possible explanation for this discrepancy is that the transformed cell lines used in these studies constitutively expressed enough NF-κB to sustain a high baseline expression of the TNF-α gene (see Figs. 2 and 6 in Ref. 10), and that other factors become rate-limiting under these conditions. Alternatively, there may be significant differences in the regulation of the mouse and human promoters. It should be noted that site κB3, the strongest NF-κB binder in the mouse promoter, is absent from the human and rabbit promoters.

NF-GMa. This factor was described by Shannon et al. (12, 13) as a ubiquitous binding activity specific for the so-called CK-1 box, which is very similar to a κB enhancer (Fig. 5) and is found upstream of a number of cytokine genes. However, the binding specificity of NF-GMa is clearly distinct from that of NF-κB (12). Our data strongly suggest that band C in the oligonucleotide EMSA (Fig. 3) and band II obtained with the −695 to −432 restriction fragment (Fig. 4) both correspond to complexes containing NF-GMa, for the following reasons: 1) an oligonucleotide containing the canonical NF-GMa binding site competed efficiently for the formation of complex C (Fig. 3) and could itself form complex C; 2) all of the oligonucleotides that were capable of forming complex C contain a CK-1 consensus sequence (Fig. 5); 3) similarly to NF-GMa, and in contrast to NF-κB, complex C could be formed with extracts from many cell types (A. N. Shakhor, unpublished observations), and was increased only marginally after activation; 4) mutation of site κB2, which has the highest affinity for the factor (8), specifically affected the formation of complex II with the −695 to −432 restriction fragment (Fig. 4).

The CK-1 motif was shown to act as a TNF, but not PMA-inducible enhancer in fibroblasts (12), and this was taken as evidence for the lack of involvement of NF-κB in the enhancer activity of CK-1. An alternative explanation would be that the CK-1 motif binds NF-κB, but requires the presence of NF-GMa to express its enhancer activity. We have not been able to generate mutations selectively affecting the binding of one or the other factor, which would provide the best test of this hypothesis. In our work, constructs containing the κB2 or κB3 enhancers were not responsive to PMA when transfected into macrophages; similarly, PMA failed to induce synthesis of TNF-α mRNA (unpublished observations). Thus, unresponsiveness to PMA does not rule out an involvement of NF-κB.

Nishizawa and Nagata (11) showed that mutations in the CK-1 motif abolished LPS-inducible expression of the mouse G-CSF promoter in a macrophage cell line, and that a portion of the G-CSF promoter containing a CK-1 motif could act as a LPS-inducible enhancer in these cells. Surprisingly, the enhancer activity seemed to be associated with sequences mapping downstream of the CK-1 site. The possibility that NF-κB may bind to the CK-1 motif of the G-CSF promoter has not been investigated, but the data in Figure 3 suggest that it may. At high concentrations, the G-CSF oligonucleotide also competed for the formation of bands A and B.

Relationship between κB and CK-1 enhancers. Figure 5 shows a comparison of the κB sites from the TNF promoter with a few other sites for which factor-binding and/or functional data are available from the literature. The sequences are arranged approximately in decreasing order of affinity for NF-κB. Several interesting features emerge from this compilation: 1) Binding of NF-κB or NF-GMa is not mutually exclusive. Apart from the sites described in this paper, a site from the granulocyte-macrophage-CSF promoter (granulocyte-macrophage-CSF2) has been shown to bind both factors, albeit by two different groups (13, 24). 2) There is no obvious way to distinguish between the two elements, as their "consensus" sequences are mutually compatible. It is likely that binding specificity is dictated at least in part by sequences flanking the consensus, and that measured enhancer activity may thus depend on the length of the oligonucleotides tested. 3) Unfortunately, only a few of these elements have been tested for LPS-inducible enhancer activity. Nevertheless, it is clear that enhancer activity is not associated uniquely with the ability to bind one or the other of the factors.

Therefore, we would argue that the CK-1 sites found in the promoters of many cytokines represent a subset of the family of κB-type enhancers, distinguished operationally by their ability to bind an additional nuclear factor, NF-GMa. It is likely that their inducible phenotype depends on a signal delivered by NF-κB. The role of NF-GMa in the expression of the TNF-α promoter remains a subject for speculation; it may act as a cofactor or an amplifier of the signal.

Other factors. Another ubiquitous protein, KBF1/H2TF1, has been shown to bind to a sequence motif very closely related to the NF-κB recognition site (25–27). KBF1/H2TF1 is thought to be a major regulator of the expression of MHC class I genes and of the associated β_2-microglobulin chain (28), and is a dimer of the 50-kD DNA-binding subunit of NF-κB (29). As has been observed for NF-GMa (12), H2TF1 should be induced upon exposure of fibroblasts to TNF, because TNF is a potent inducer of MHC class I expression in these cells (30). NF-GMa and KBF1/H2TF1 have not been compared directly so far, although a examination of published EMSA patterns obtained with the two factors would suggest that they are distinct. Preliminary data using a KBF1-specific antiserum generously provided by A. Israël (Inst. Pasteur, Paris, France) suggest that complex B (the "constitutive" form of NF-κB) contains KBF1, whereas complex A (the "inducible" form) contains heterodimeric NF-κB.

The oligonucleotide containing the TNF-α Y box forms an abundant complex with whole cell extracts from a variety of cell types. Similarly, the −316 to −161 restriction fragment forms a complex (Fig. 4, *arrow*) that can be inhibited by a Y box oligonucleotide (7) or by a mutation in the Y box. The decrease in LPS inducibility upon 5′ terminal deletion of the Y box and an adjacent CA_n microsatellite, and the increase in a Y box-specific complex upon LPS stimulation was taken as evidence for an involvement of the Y box in the inducibility of the promoter (7). From the data presented here, it is obvious that the Y box does not act as a LPS-inducible enhancer, and that mutation or partial deletion of the Y box has no effect on the promoter. This result is compatible with the view that CCAAT box family elements affect the baseline activity of promoters, rather than their inducibility. Our earlier data may then suggest an enhancer activity for the microsatellite, as has been observed in other systems (31).

Conclusions. It is clear that the combination of cis-acting elements and nuclear factors studied in this report represent only a very small proportion of potential DNA-protein interactions in the TNF-α promoter, and that in all probability many more remain to be described. However, it is remarkable that the substitution of as few as 11 nucleotides out of the more than 1000 included in TNF(−1059)CAT reduced the inducibility of the promoter by 10-fold (quadruple mutant in Table II). This leads us to believe that we have characterized most of the elements that are directly involved in the LPS inducibility of the promoter. All of these elements are capable of binding NF-κB. This apparent redundancy in elements that respond to the same signal and apparently act in a cooperative way may increase the sensitivity and the dynamic range of the transcriptional response to LPS. The potential role of other factors binding to the same elements remains to be determined.

The data presented here provide additional evidence for the central role of NF-κB in the transduction of activation signals to the nucleus of leukocytes, in particular those signals affecting the synthesis of cytokines. Although it is impossible at this point to assess the importance of factors that act independently of NF-κB, there is every reason to believe that NF-κB has a role to play in the transcriptional activation of many cytokine genes.

Acknowledgments. We thank Martine Collart, Markus Nabholz, and Sergei Nedospasov for communicating their results before publication, for many useful discussions, and for the generous gift of reagents. We are grateful to Jean-Charles Cerottini for his critical reading of the manuscript and for his continuing support. Andrée Porret provided expert technical assistance.

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0026-895X/96/050860-14$3.00/0

MOLECULAR PHARMACOLOGY, 49:860–873 (1996).

Inhibitory Effect of E3330, a Novel Quinone Derivative Able to Suppress Tumor Necrosis Factor-α Generation, on Activation of Nuclear Factor-κB

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SUMMARY

(2*E*)-3-[5-(2,3-Dimethoxy-6-methyl-1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid (E3330), is a novel agent with hepatoprotective activity. We report the effect of E3330 on transcriptional activation of tumor necrosis factor (TNF)-α gene and on nuclear factor (NF)-κB activation. Nuclear run-on experiments showed that E3330 decreases transcriptional activation of TNF-α gene induced by lipopolysaccharide (LPS) stimulation in human peripheral monocytes. To investigate the inhibitory mechanisms, we constructed a secreted-type placental alkaline phosphatase (PLAP) reporter gene whose transcription is controlled by a 1.4-kb human TNF-α promoter. A stable transformant of the PLAP reporter gene derived from human monocytic cell line showed very little activity of the promoter before stimulation, whereas LPS stimulation led to a dramatic increase in PLAP activity. E3330 inhibited this induced promoter activity in a dose-dependent manner. There are four putative NF-κB binding sites (κB-1, κB-2, κB-3, κB-4) in human TNF-α promoter. By using mutated promoter-PLAP plasmids, we established that these NF-κB sites were necessary for induction of TNF-α transcription on stimulation with LPS. A gel retardation experiment with synthetic double-stranded oligonucleotides showed that activated NF-κB consisting of p50/p65 heterodimer bound to all four putative NF-κB DNA probes, suggesting that all four putative NF-κB recognition sites play an important role in inducible TNF-α expression. E3330 decreased activated NF-κB in nuclei, suggesting that E3330 inhibits NF-κB activation and/or translocation to the nuclei. Western blotting analysis with anti-IκB-α antibody indicated that E3330 inhibited degradation of IκB-α, which is an inhibitory protein of NF-κB, in LPS-stimulated monocytes. E3330 may suppress the production of active oxygen species serving as common messengers to activate NF-κB.

Monocytes/macrophages play significant roles in inflammatory and immune responses. When monocytes/macrophages are stimulated with various stimuli, such as LPS, PMA, and IL-1, the cells generate cytokines, such as TNF-α, IL-1α, IL-1β, IL-6, and IL-8, which mediate a wide range of biological activities (1). Among these cytokines, TNF-α shows various activities, including induction of pyrogen, activation of macrophages and neutrophiles, induction of expression of adhesion molecules on endothelial cell membrane, enhancement of fibroblast growth, and activation of HIV-1 expression (2, 3). Recently, it was proposed that TNF-α is involved in the pathogenesis of human liver injury, such as fulminant hepatic failure, alcoholic hepatitis, and chronic liver disease (4–6). Therefore, an inhibitor of TNF-α generation could be a useful therapeutic drug in the treatment of these diseases.

E3330 was reported to inhibit LPS-induced TNF-α generation in human monocytes, rat resident peritoneal macrophages, and *Propionibacterium*-elicited peritoneal macrophages, rat Kupffer cells and rat spleen macrophages (7). A therapeutic effect of E3330 and an inhibitory effect of E3330 on *in vivo* TNF-α generation in plasma in mice with endotoxin-mediated hepatitis and in rats with galactosamine-induced hepatitis were also reported (8, 9). Northern blot analysis indicated that the inhibitory effect of E3330 on TNF-α generation is due to inhibition of mRNA biosynthesis and/or destabilization of mRNA.

Biosynthesis of TNF-α mRNA is regulated at transcriptional and post-transcriptional levels. Binding of NF-κB to NF-κB recognition sites in the TNF-α gene 5′ upstream region is important for transcription of human and murine TNF-α gene (10–12). NF-κB is a critical regulator of several genes involved in immune and inflammatory responses (13,

ABBREVIATIONS: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; IL, interleukin; IκB, inhibitor of NF-κB; HIV-1, human immunodeficiency virus type-1; LTR, long terminal repeat; PLAP, human placental alkaline phosphatase; AIDS, acquired immune deficiency syndrome; SDS, sodium dodecyl sulfate; PMA, phorbol-12,13-myristate acetate; fMLP, *N*-formyl-MET-LEU-PHE; E3330, (2*E*)-3-[5-(2,3-dimethoxy-6-methyl-1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid; HBSS, Hanks' balanced salt solution; NP-40, Nonidet-40; bp, base pair(s); SSC, standard saline citrate; BGH, bovine growth hormone; TK, thymidine kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

14) and was first identified as a nuclear factor that activates the expression of the κ light chain gene. Soon, it became evident that this factor binds NF-κB recognition sequences (e.g., 5′-GGGACTTTCC-3′) that exist in the 5′ upstream region of cytokine, cytokine receptor, and virus genes. NF-κB transcription complexes are dimeric proteins consisting of one or two members of a family related to the c-Rel proto-oncogene, such as NF-κB1 (p105, p50), NF-κB2 (p100, p52), NF-κB (p50/p65), Rel, RelA (p65), RelB, dorsal, Dif, and Cif. NF-κB p50/p65 heterodimer is present in the cytosol of resting cells as the bound form with an inhibitory protein, IκB-α. When the cells are stimulated with various agents such as LPS, PMA, and TNF-α, the cytosolic NF-κB/IκB-α complex is dissociated and free NF-κB translocates to the nuclei.

To clarify the mechanism of the inhibitory effect of E3330 on TNF-α gene expression, we examined the effects of E3330 on the transcriptional activation of TNF-α gene and on the activation of NF-κB.

Materials and Methods

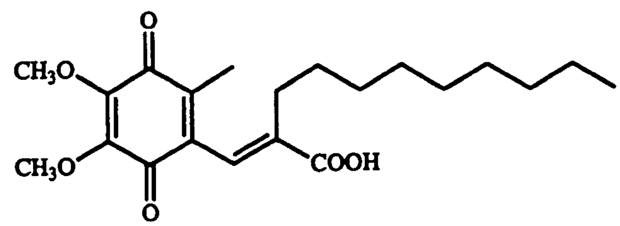
Cells

THP-1 (acute monocytic leukemia human) and Raw264.7 (macrophage, Abelson leukemia virus-transformed, BALB/c) were obtained from the American Type Culture Collection (Rockville, MD).

Drugs and Chemicals

LPS (*Escherichia coli* 0127:B08), TRI zol, polydeoxyinosinic-deoxycytidylic acid, cytochrome *c*, superoxide dismutase, catalase, PMA, fMLP, xanthine, xanthine oxidase, Triton X-100, NP-40, deoxycholate, chymostatin, pepstatin A, NaF, Na_3VO_4, MTT, dithiothreitol, geneticin (G418), and PMSF were purchased from Sigma Chemical Co. (St. Louis, MO); RPMI 1640, HBSS without Ca^{2+} and Mg^{2+}, penicillin/streptomycin, and HEPES, GIBCO-BRL (Grand Island, NY); Ficoll-Paque, NAP-5 columns, ultrapure NTP set, and CellPhect Transfection Kit (DEAE-dextran), Pharmacia (Uppsala, Sweden); aprotinin and leupeptin, Boehringer Mannheim Yamanouchi (Tokyo, Japan); NF-κB (p50, human), Promega Co. (Madison, WI); endotoxin-free fetal calf serum, Nippon B.M.A. Co. (Osaka, Japan); [α-^{32}P]dCTP (220 TBq/mmol), [α-^{32}P]UTP (110 TBq/mmol), anti-rabbit Ig horseradish peroxidase-linked whole antibody (from donkey), Hybond-ECL (nitrocellulose), and Hyperfilm-ECL and ECL Western blotting detection reagents, Amersham (Arlington Heights, IL); human placenta cDNA library (HL1075b), genomic library (HL1067J), and the vector pTKβ, Clontech Laboratories (Palo Alto, CA); vent DNA polymerase, New England Biolabs, Inc. (Beverly, MA); pRC/CMV, Invitrogen Corp. (San Diego, CA); anti-p50 antibody, anti-p52 antibody, anti-p65 antibody, anti-Rel antibody, anti-RelB antibody, and anti-IκB-α antibody, Santa Cruz Biotechnology (Santa Cruz, CA); Klenow fragment, Takara Shuzo Co. (Kyoto, Japan); restriction enzymes and DNA modifying enzymes, New England Biolabs, Toyobo KK (Osaka, Japan), or Takara Shuzo Co.; EDTA and EGTA, Nacalai Tesque (Kyoto, Japan); Tris, 40% acrylamide/bis solution (19:1), 10× Tris/boric acid/EDTA buffer (1× = 89 mM Tris, 89 mM boric acid, and 2 mM EDTA), ammonium persulfate, glycine, and boric acid, Bio-Rad Laboratories (Richmond, CA); 20× SSC (1× = 150 mM NaCl and 15 mM sodium citrate), reaction buffer building set, and glycerol, Wako Pure Chemical Industries, Ltd. (Osaka, Japan); T7-GEN *in vitro* mutagenesis kit, United States Biochemical Corp. (Cleveland, OH); Multigel 4/20, Daiichi Pure Chemicals Co. (Tokyo, Japan); and Smilight (Lumistain; 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2′-adamantane]), Sumitomo Kinzoku (Tokyo, Japan).

E3330 (Fig. 1) was synthesized in our laboratories. DNA probes of human TNF-α NF-κB-like sites and HIV-1 NF-κB site were synthesized according to a solid-phase phosphate triester method with a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA).



E3330

(2E)-3-[5-(2,3-dimethoxy-6-methyl-1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid

Fig. 1. Chemical structure of E3330.

Nuclear Run-on Assay

Human monocytes from peripheral blood of healthy male volunteers were obtained through the method previously reported (15). Briefly, human mononuclear cells were isolated through Ficoll-Paque density gradient sedimentation. The cells were suspended at a concentration of 2 × 10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated autologous serum, seeded at 20 ml/dish onto 150-mm dishes (Falcon 3025, Becton Dickinson, NJ), and cultured for 1.5 hr. Nonadherent cells were removed through rinsing, and the remaining cells were used as the monocyte preparation. The monocytes were cultured in the presence or absence of E3330 for 30 min, followed by stimulation with 100 ng/ml LPS for 30 min. Then, the cells were rinsed free of HBSS and lysed in NP-40 lysis buffer (10 mM Tris·HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40), and the nuclei were isolated by spinning for 5 min in a microfuge. The nuclei were resuspended in 100 μl of glycerol buffer (50 mM Tris·HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), frozen in liquid N_2, and stored at −80°. For the nuclear run-on assay, isolated nuclei were mixed with an equal volume of reaction buffer (10 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 1 mM concentrations of ATP, GTP, and CTP, and 37 MBq of [α-^{32}P]UTP) and incubated at 30° for 30 min. RNA was extracted with TRI zol and purified through ethanol precipitation. For the preparation of filters, both purified human TNF-α cDNA and β-actin cDNA were used. These fragments were denatured with 0.1 M NaOH at room temperature for 30 min. After the addition of an equal volume of 6× SSC, the mixture was heated at 95° for 10 min and quickly chilled on ice. The solution containing DNA fragments was filtered through nitrocellulose with use of a slot-blot manifold. Each slot contained 5 μg of DNA. The filter was baked at 80° for 2 hr, prehybridized in 6× SSC at 42°, and hybridized to 0.75–1 × 10^6 cpm [^{32}P]RNA preparation in 6× SSC at 42° for 2 days. After the hybridization, the filter was washed three times in 2× SSC/0.1% SDS at room temperature and twice in 0.2× SSC/0.1% SDS and then exposed to an X-ray film for 3 days.

Construction of DNA Coding Secreted Form of PLAP

Human PLAP cDNA has been sequenced (16), and its modification to secreted-type PLAP (truncation of the carboxyl-terminal 24 amino acids) was also reported (17). We isolated the 1276-bp 5′ part of this cDNA through polymerase chain reaction from a human placenta cDNA library (HL1075b). Two sets of primers (A, 5′-CCAGAATTC-CTGCCTCGCCACTGTCC-3′ and 5′-TTAGGATCCTGGCAGCTGT-CAC-3′; B, 5′-GTGACAGCTGCCAGGATCCTAA-3′ and 5′-AGGAC-CGTGTAGGCCTCCCTGT-3′) gave two overlapping DNA fragments

(273 and 1028 bp, respectively). Digestion of these DNA fragments with restriction enzyme *Bam*HI and ligation with *Bam*HI cohesive ends yielded the 1276-bp 5′ part of the PLAP-coding DNA. The isolated 1276-bp fragment was inserted into pBlueScript KS plasmid with the use of the *Eco*RI and *Sma*I restriction sites that are located at the 5′ end and 22 bp upstream of the 3′ end of this fragment. The nucleotide sequence of this 1276-bp insert was identical to the reported cDNA sequence (16).

The 3′ part of PLAP-coding DNA was not amplified by polymerase chain reaction, due to its GC-rich sequence. We designed an artificial DNA that codes 108 amino acid residues [amino acid residues 382–489 in Millan (16)], has a translational stop codon TAA instead of the Arg^{490} codon, and has a restriction enzyme recognition site for *Bgl*II in its 3′ end. We synthesized two pairs of 98–100 base oligonucleotides (Fig. 2; model 394 DNA/RNA synthesizer, Applied Biosystems). Oligonucleotides of each pair were mixed, annealed via the 17-nucleotide complementary sequence of the 3′ ends, and subjected to polymerase chain reaction (vent DNA polymerase) to yield double-stranded DNA segments (179 and 181 bp, respectively). These two DNA fragments were subcloned in the *Hin*cII site of pUG131 [multilinker of pUC18 was replaced with that of M13tg131 vector (18)], and their nucleotide sequences were confirmed with the dideoxy termination method. Plasmids with the correct sequence were digested with *Xma*I and *Aat*II or *Aat*II and *Bgl*II to yield *Xma*I/*Aat*II 165-bp fragment and *Aat*II/*Bgl*II 164-bp fragment, respectively. These two fragments were purified on agarose and inserted between the *Xma*I and *Bgl*II sites of pUG131. The 329-bp insert of this plasmid was cut out with *Xma*I and *Bgl*II and ligated with 1276-bp *Hin*dIII/*Xma*I fragment (the *Hin*dIII site is located in the multilinker site of KS plasmid). The resulting 1593-bp PLAP-coding DNA was inserted between the *Hin*dIII and *Bgl*II sites of pUG131. The 5′ noncoding region and 15 bp of coding DNA were cut out with *Eco*RI and *Sph*I, and then *Eco*RI/*Hin*dIII/*Sph*I[d] adapter (5′-pAATTCAAGCTTACCATG-3′ and 5′-pGTAAGCTTG-3′) was inserted into the deletion site. This plasmid contains DNA coding the secreted form of PLAP (1548 bp, *Hin*dIII to *Bgl*II) and is named pUG-PLAP (Fig. 3A).

```
A

   A  R  D  R  K  A  Y  T  V  L  L  Y  G  N  G  P  G  Y  V  L     401
5'-AAGCCCGGGATCGTAAGGCCTACACAGTGCTACTGTATGGCAATGGCCCAGGGTATGTCCTA
     XmaI

   K  D  G  A  R  P  D  V  T  E  S  E  S  G  S  P  E  Y  R  Q     421
   AAGGATGGAGCTAGACCAGATGTCACAGAGTCAGAG-3'
                  3'-TACAGTGTCTCAGTCTCAGACCTAGAGGTCTCATGGCAGTC

   Q  S  A  V  P  L  D  E  E  T  H  A  G  E  D  V  A  V  F        440

   GTTAGCCGGCATGGCAATCTACTTCTCTGAGTGCGTCCCCTTCTGCAGCGACAGAAA-5'
                                              AatII

B

   E  D  V  A  V  F  A  R  G  P  Q  A  H  L  V  H  G  V  Q  E     454
5'-GGAAGACGTCGCTGTCTTTGCAAGAGGTCCCCAGGCACATCTCGTGCATGGCGTACAGGAA
       AatII

   Q  T  F  I  A  H  V  M  A  F  A  A  C  L  E  P  Y  T  A  C     474
   CAGACTTTCATCGCTCATGTAATGGCATTCGCAGCAT-3'
                  3'-TTACCGTAAGCGTCGTACAAACCTCGGTATATGGCGAACG

   D  L  A  P  P  A  G  T  T  D  A  A  H  P  G  *                 489

   CTAAATCGAGGTGGTCGTCCATGCTGTCTGCGACGGGTAGGTCCAATTGGGTCTAGACCT-5'
                                                        BglII
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Fig. 2. Nucleotide sequences of the synthetic oligonucleotides for the 3′ end of PLAP-coding DNA. Two pairs of artificial 98–100-mer oligonucleotides were synthesized. The nucleotide sequences shown in A and B code 59 and 55 amino acid residues, respectively [corresponding to amino acid residues 382–489 in Millan (16); *top row*]. *, Stop codon.

Construction of PLAP Reporter Plasmids

pSV2-PLAP. pSV2-PLAP was derived from pSV2-dhfr plasmid (19). PLAP-coding DNA was cut out from pUG-PLAP through digestion with *Hin*dIII and *Bgl*II, and the 1.5-kb DNA fragment was ligated to the large fragment of pSV2-dhfr plasmid cut with the same enzymes to produce pSV2-PLAP (Fig. 3B). PLAP gene expression is controlled by the SV40 early promoter in this vector.

pUG-BGH-PLAP. To minimize read-through transcription, BGH terminator was introduced in the upstream region of the reporter expression cassette. Digestion of pRC/CMV with *Sac*I and *Sph*I gave the 217-bp BGH terminator. This fragment was ligated with pUG-131 that had been cut with *Sac*I and *Sph*I to generate pUG-BGH. A DNA fragment that has the PLAP coding region, SV40 splice sites, and a $poly(A)^+$ signal (2.5 kb) was isolated from pSV2-PLAP by cutting completely with *Hin*dIII and then partially with *Bam*HI. This 2.5-kb fragment was ligated with pUG-BGH that had been cut with *Hin*dIII and *Bam*HI to give pUG-BGH-PLAP (Fig. 3C).

TNF-α-PLAP. The complete DNA sequence of human lymphotoxin and TNF-α gene locus (7 kb) has been reported (20). A 1.4-kb TNF-α promoter region DNA (−1211 to +167 relative to TNF-α cap site) was isolated as three overlapping DNA fragments by polymerase chain reaction from a genomic library (HL1067J, Clontech Laboratories). Three pairs of primers (A, 5′CGATCTAGAACTTCCCAGTCTATCTAAG-3′ and 5′-TTTTCATGAAGCTCTCACTTCTC-3′; B, 5′-GAGAAGTGAGAGCTTCATGAAAA-3′ and 5′-CTTTTGGGGACCAGGTCTGTG-3′; C, 5′-CACAGACCTGGTCCCCAAAAG-3′ and 5′-TAGAAGCTTTCCAGGGGAGAGAGGGTG-3′) gave a 470-bp *Xba*I/*Bsp*HI fragment, a 382-bp *Bsp*HI/*Bst*XI fragment, and a 524-bp *Bst*XI/*Hin*dIII fragment, respectively. These three fragments were inserted into pUG-BGH-PLAP that had been cut with *Xba*I and *Hin*dIII. The resulting plasmid, named TNF-α-PLAP (Fig. 3D), was sequenced, and seven nucleotides were found to be different than the reported sequence (+146/C, −315/A, −448/delete, −511/insertion A, −575/C, −655/A, and −892/G).

Mutated TNF-α-PLAP. Putative NF-κB recognition sequences in the TNF-α 5′ upstream enhancer region (κB-1, −634 to −625; κB-2, −605 to −596; κB-3, −220 to −211; κB-4, −105 to −96) were mutated with the use of a T7-GEN *in vitro* mutagenesis kit. A 1.4-kb TNF-α enhancer/promoter region was subcloned in M13 mp18 and mp19 plasmids to produce a single-stranded template for *in vitro* annealing of mutagenic primers (for κB-1, 5′-CACCCCCGGGAATTTATAGACCCCACTGGGG-3′; for κB-2, 5′-CAAGCCTGGGACAGTTTCGGGGAGTGAAATC-3′; for κB-3, 5′-AGCATCAAGGATAGTTTTCACACTCCCCATC-3′; for κB-4, 5′-CCAGATGAGCTCATAAATTTCTCCACCAAGG-3′) and synthesis of the complementary strand. Correct mutations were selected through sequencing and then introduced into pUG-BGH-PLAP reporter plasmid as *Xba*I/*Hin*dIII fragments.

HIV-1 κB-PLAP. For convenience in inserting multicopy enhancer elements, a *Spe*I site was introduced into the pUG-BGH-PLAP. pUG-BGH-PLAP was digested with *Sph*I and *Xba*I and then ligated with *Sph*I/*Spe*I/*Xba*I adapter (5′-pCACTAGTAAAT-3′ and 5′-pCTAGATTTACTAGTGCATG-3′) to yield pUG-BGH-PLAP (*Spe*I). Truncated herpes simplex virus TK promoter (−51 to +105 from the cap site of TK) was isolated through polymerase chain reaction (21, 22). Primers (5′-GGATCTAGACCCCGCCCAGCGTCTTGTCA-3′ and 5′-GGAAAGCTTGCGGCACGCTGTTGACGCTG-3′) that have *Xba*I or *Hin*dIII sites in their 5′ ends were used to amplify the 157-bp TK promoter fragment from the vector pTKβ. After digestion with *Xba*I and *Hin*dIII, 157-bp *Xba*I/*Hin*dIII fragment was ligated to pUG-BGH-PLAP (*Spe*I) that had been cut with the same enzymes to yield TK-PLAP. The HIV-1 κB-PLAP (Fig. 3E) construct contains four copies of the synthetic oligonucleotides 5′-pCTAGT*GGGACTTTCC*T-3′ and 3′-A*CCCTGAAAGG*AGATCp-5′, corresponding to the 10-bp NF-κB-responsive element (−103 to −94 in HIV-1 LTR. Italics show NF-κB sequences.) inserted upstream of a truncated

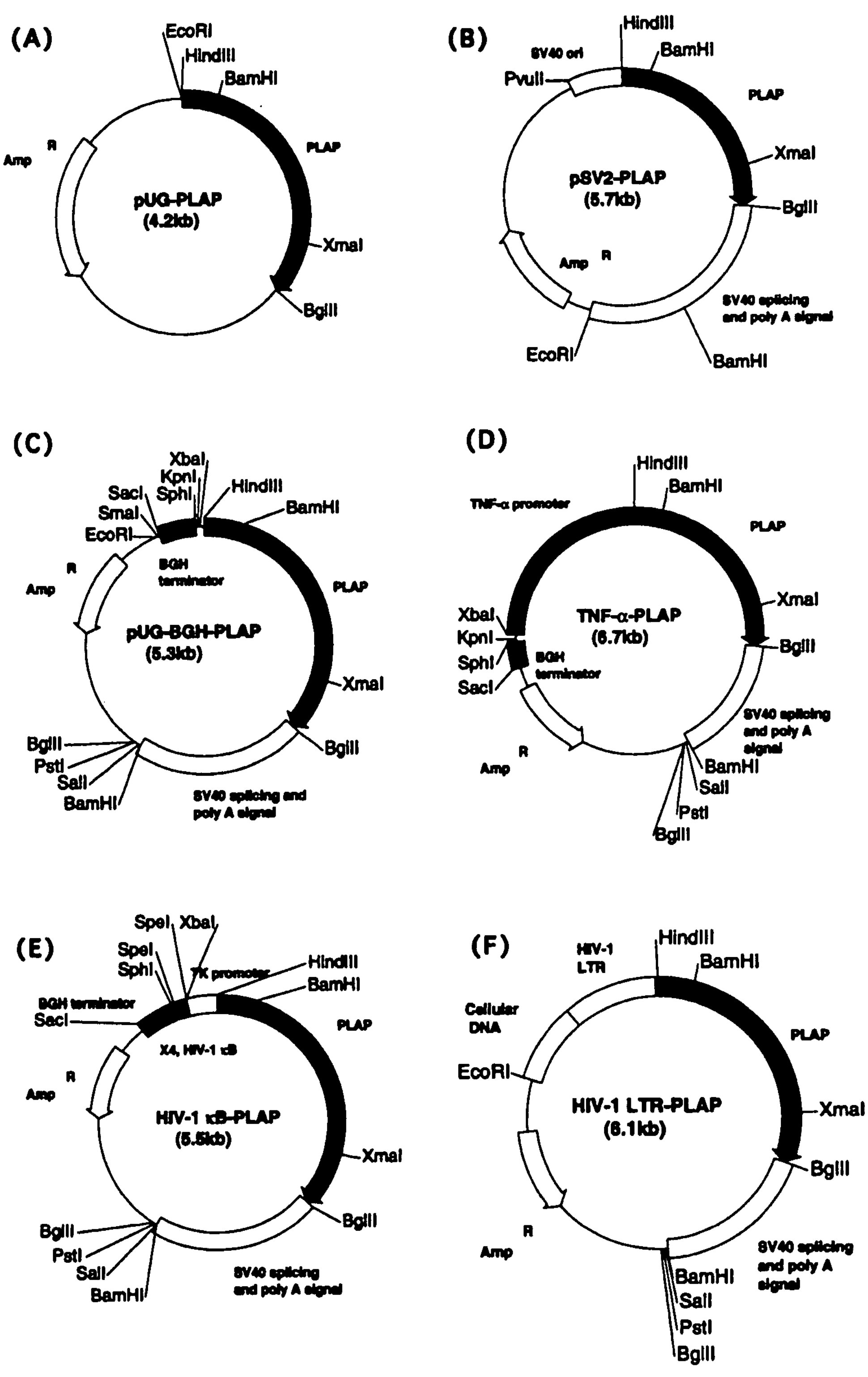

Fig. 3. Structure of plasmid vectors containing secreted human PLAP. *Solid arrow of each plasmid*, a 1.5-kb fragment of secreted form of human PLAP that extends from *Hin*dIII to *Bgl*II. The *open area* from *Pvu*II to *Hin*dIII in pSV2-PLAP (B) is the SV40 sequence that contains the SV40 origin of replication and the early promoter. Other *open areas* from *Bgl*II to *Bam*HI (or *Eco*RI) in pSV2-PLAP (B), pUG-BGH-PLAP (C), TNF-α-PLAP (D), HIV-1 κB-PLAP (E), and HIV-1 LTR-PLAP (F) are SV40 sequences encoding the intron for small t antigen and the polyadenylation signal for early mRNA. The *lightly stippled areas* in pUG-BGH-PLAP (C), TNF-α-PLAP (D), and HIV-1 κB-PLAP (E) are a 217-bp fragment of BGH terminator that extends from *Sac*I to *Sph*I. The *heavily stippled area* in TNF-α-PLAP (D) is a 1.4-kb TNF-α promoter fragment. The *open area* in HIV-1 κB-PLAP (E) is 157 bp of truncated TK promoter segment that extends from *Spe*I/*Xba*I to *Hin*dIII. The *solid segment* from *Spe*I to *Spe*I/*Xba*I in HIV-1 κB-PLAP (E) contains four copies of HIV-1 NF-κB element. The *open area* in HIV-1 LTR-PLAP (F) is the HIV-1 proviral 5′ LTR sequence (531 bp).

TK promoter in TK-PLAP through the use of restriction sites *Spe*I and *Xba*I. The orientation of NF-κB-responsive elements was found to be forward, forward, backward, and backward relative to that in HIV-1 LTR by sequencing.

HIV-1 LTR-PLAP. HIV-1 LTR-containing DNA fragment was isolated from pUC-BENN-chloramphenicol acetyltransferase plasmid (Dr. M. A. Martin, provided through the National Institute of Allergy and Infectious Diseases, AIDS Research and Reference Reagent Program) (23). pUC-BENN-chloramphenicol acetyltransferase plasmid was cut with *Eco*RI and *Hin*dIII, and then a 981-bp fragment containing cellular DNA and HIV-1 LTR was purified and ligated to pUG-BGH-PLAP to yield HIV-1 LTR-PLAP (Fig. 3F).

Preparation of TR-1 Cells through Stable Transfection of TNF-α Reporter Plasmid into Genome of THP-1 Cells

To produce stable reporter cells responsive to LPS stimulation, a 1.2-kb *SalI/XhoI* fragment of the PGK-neo expression cassette was introduced into the *SalI* site of TNF-α-PLAP plasmid (24). The resulting plasmid TNF-α-PLAP-PGK-neo was transfected into THP-1 cells through electroporation (Bio-Rad Gene Pulser), and the cells were subjected to G418 (1 mg/ml) selection in 96-well plates. The G418-resistant clones were isolated to check their LPS responsiveness. One clone (TR-1) produced PLAP enzyme and secreted it into the culture medium on stimulation with LPS.

Stimulation of TR-1 with LPS

TR-1 cells were maintained in RPMI 1640 containing 10% heat-inactivated endotoxin-free fetal calf serum and G-418 (1 mg/ml). The cells, in RPMI 1640 medium without G418, were seeded at a density of 1.0×10^5 cells/well onto 48-well plates (Costar 3548, Costar Corp., Cambridge, MA). The TR-1 cells were cultured in the presence or absence of E3330 for 30 min, followed by stimulation with 100 ng/ml of LPS for 18 hr. After the stimulation, culture supernatant was drawn from each well and assayed for alkaline phosphatase activity (described below).

Transfections and PLAP Assay

Cells of Raw264.7, a mouse macrophage cell line, in RPMI 1640 containing 10% heat-inactivated, endotoxin-free fetal calf serum were seeded before transfection at a density of 1.5×10^6 cells/well onto six-well plates (Falcon 3046, Becton Dickinson, NJ). These cells were transfected with PLAP reporter plasmid (15 μg) through the DEAE-dextran method (25). At 16 hr after transfection, cells (5×10^6 cells/well, six-well plate) were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with 1 μg/ml of LPS for 48 hr. After the stimulation, culture supernatant was drawn from each well and assayed for alkaline phosphatase (described below). Alkaline phosphatase activity secreted into the culture medium was quantified with the use of a chemiluminescent substrate, 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2'-adamantane] (Lumistain reagent) (26). To inactivate tissue-nonspecific alkaline phosphatase that was contained in the culture medium, samples were heated at 65° for 30 min before measurements were made. Aliquots of 10 μl were mixed with 90 μl of assay buffer (0.28 M Na_2CO_3-$NaHCO_3$, pH 10.0, containing 8.0 mM $MgSO_4$) in a microplate (Dynatech, Chantilly, VA), and then 100 μl of Lumistain reagent was added and mixed. After 60 min at room temperature, steady state chemiluminescence was measured with a microplate luminometer (LB96P, EG&G Bertold, Bad Wildbad, Germany).

Preparation of Nuclear Extracts and Gel Shift Assay

Human monocytes were separated from peripheral blood according to the method described above. The cells were suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated autologous serum, seeded at 10 ml/dish onto 100-mm dishes (Costar 3100), and cultured for 1.5 hr. Nonadherent cells were removed by rinsing, and the remaining cells were used as the monocyte preparation. The monocytes were cultured in the presence or absence of E3330 for 30 min, followed by stimulation with 10 ng/ml of LPS for 2 hr. Then, the nuclear extracts were isolated according to the known rapid preparation method with a slight modification (27). Briefly, cells were collected with a cell scraper, washed with 1 ml of HBSS, and pelleted by spinning for 10 min in a microfuge. The cell pellet was resuspended in 100 μl of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF). The cells were allowed to swell on ice for 15 min, mixed with 10 μl of 10% solution of NP-40, and vigorously vortexed for 10 sec. The cell homogenate was centrifuged for 10 min in a microfuge. After removal of the supernatant, the nuclear pellets were resuspended in 25 μl of buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF), and the tube was vigorously shaken at 4° for 15 min on a shaking platform. The nuclear extracts were centrifuged for 10 min in a microfuge, and the supernatant was frozen in aliquots at −80°.

The NF-κB probes of TNF-α used for gel shift assay consisted of the NF-κB-like sites located at −634 bp (κB-1, 5'-GG-GTCT *GT-GAATTCCC*GGGGGTGA-3'), −605 bp (κB-2, 5'-GG-CTCCCC *GGGGCTGTCCC*AGCT-3'), −220 bp (κB-3, 5'-GG-TGTGA *GGGG-TATCCTT*GATGCT-3'), and −105 bp (κB-4, 5'-GG-CTCAT *GGGTT-TCTCC*ACCAAGG-3') from the transcription start point of TNF-α gene (20) (italics show NF-κB-like sequences). The NF-κB probe of HIV-1 used for gel shift assay consisted of the NF-κB site located between −103 and −90 (5'-GG-CTACAA *GGGACTTTCCGCT GGG-GACTTTCC*AGG-3') nucleotides from the transcription start point of HIV-1 (28) (italics show NF-κB sequences). Double-stranded oligonucleotides were labeled with [α-^{32}P]dCTP with use of the Klenow fragment. The labeled DNA probes were purified with use of NAP-5 columns. Gel shift assay was performed according to the previously described method with a slight modification (29). The nuclear extracts (2 μg/ml) were incubated with ^{32}P-labeled NF-κB probe of TNF-α or HIV-1 (10,000–20,000 cpm) in the binding buffer (10 mM Tris·HCl, 40 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1% NP-40, 1% deoxycholate, 3 μg/ml polydeoxyinosinic-deoxycytidylic acid) at room temperature for 30 min. DNA/protein complexes were separated from free DNA probes on native 5% polyacrylamide gel. The gels were vacuum-dried and exposed to an X-ray film or an imaging plate of the BAS 2000 system (Fuji Photo Film Co., Tokyo, Japan).

To characterize the bands, we carried out supershift assay. The nuclear extract and oligonucleotide were mixed, specific antibody (3 μg) against nuclear factor was added, and the mixture was incubated at 4° for 1 hr; then, gel shift assay was conducted according to the procedure described above.

To examine the effect of E3330 on direct binding of NF-κB protein to NF-κB probes, 2 μg of nuclear extract was isolated from human monocytes that had been stimulated with 10 ng/ml of LPS for 2 hr, or 2 μg of the human recombinant p50 protein was incubated with TNF-α or HIV-1 κB probe in the presence or absence of E3330 for 30 min at room temperature.

Western Blotting Analysis of IκB-α Degradation

The monocytes attached to 150-mm dishes were cultured in the presence or absence of E3330 for 30 min, followed by stimulation with 100 ng/ml of LPS for 30 min. After the stimulation, the cells were rinsed with HBSS and then lysed with 200 μl of ice-cold lysis buffer (0.2 M HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 0.1 mM leupeptin, 1 mM PMSF, 1 mM Na_3VO_4, 50 mM NaF). The lysed samples were centrifuged for 10 min in a microfuge at 4° to remove insoluble debris. Then, 50 μl of 5-fold-concentrated Laemmli buffer and 10 μl of 2-ME were added, and the entire mixture was boiled for 3 min. The resulting samples (20 μl) were analyzed with electrophoresis on 10–20% Tris-glycine-buffered polyacrylamide gradient gels in SDS. The separated proteins were transferred to 0.22-μm nitrocellulose membrane with an electroblotter. The membrane was analyzed for the degradation of IκB-α proteins with rabbit anti-IκB-α antibody and horseradish peroxidase-linked donkey anti-rabbit IgG antiserum. The membranes were dried, incubated in ECL reagents, covered with Saran wrap, and exposed to ECL hyperfilms.

Superoxide Anion Production

Human monocytes were separated from peripheral blood through the method described above. The cells were suspended at a concentration of 1×10^7 cells/ml in RPMI 1640 containing 10% heat-inactivated autologous serum, seeded at 100 μl/well onto 96-well plates (Falcon 3092), and cultured for 1.5 hr. After removal of nonadherent cells through rinsing, the reaction buffer [HBSS (−) containing 0.1% glucose and 25 μg/ml catalase, in the

presence or absence of 25 μg/ml of superoxide dismutase] was added to the monocytes. The monocytes were incubated in the presence or absence of E3330 for 30 min, followed by the addition of 80 μM cytochrome *c* and stimulation with PMA (10 nM), LPS (1 μg/ml), or fMLP (10 μM) for 60 min in the final volume of 200 μl.

Superoxide anion-scavenging activity of E3330 was determined in a cell-free system. Xanthine oxidase (10 munits/ml) and xanthine (100 μM) were incubated in the presence or absence of a test compound for 30 min.

The amount of superoxide anion production was measured in terms of superoxide dismutase-inhibitable cytochrome *c* reduction with an automatic microplate reader (EL 340I, Bio-Tek Instruments, Winooski, VT).

Cell Viability

Cell viability was tested with the MTT colorimetric method (30) or lactate dehydrogenase assay.

Results

Inhibitory effect of E3330 on transcriptional rate of TNF-α gene. Northern blot analysis for TNF-α mRNA indicated that the inhibitory effect of E3330 on TNF-α generation is due to inhibition of TNF-α mRNA biosynthesis (7). To confirm this, nuclear run-on experiments were performed. The transcription rate of TNF-α gene in human monocytes immediately rose after LPS (100 ng/ml) stimulation and reached a maximum at 30 min. Based on densitometry with a BAS 2000 image analyzer, the increase of TNF-α gene transcription over the basal level reached 12.0-fold. Pretreatment with E3330 decreased the transcription rate of TNF-α gene in dose-dependent manner but did not significantly affect transcription of actin mRNA (Fig. 4A). Image analysis indicated that the inhibition by E3330 at 30 and 100 μM amounted to 65% and 85%, respectively (Fig. 4B). At 100 μM, E3330 did not show measurable cytotoxicity in the MTT assay. These results indicate that LPS stimulation increases the TNF-α gene transcription rate and E3330 inhibits this transcriptional activation.

Inhibitory effect of E3330 on transcriptional activation of TNF-α gene. To assess the inhibitory effect of E3330 on the transcriptional activation of TNF-α gene, we performed the assay with TNF-α-PLAP reporter gene. We used the expression system of secreted-type PLAP gene as a reporter because the product of PLAP gene was secreted into the culture medium and could be detected sensitively with a chemiluminescent substrate, 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2′-adamantane] (26).

As shown in Fig. 5A, Raw264.7 cells transiently transfected with PLAP reporter plasmids containing 1.4-kb TNF-α promoter showed very little PLAP activity before stimulation. However, stimulation of the cells with LPS led to a ~10-fold increase in PLAP activity. In the presence of E3330, this inducible expression was inhibited. Inhibition by 30 μM E3330 amounted to 54%, and this concentration of E3330 did not show any cytotoxicity as measured with MTT assay.

Next, we prepared a stable transformant of THP-1 cell (TR-1) in which the TNF-α-PLAP reporter gene was integrated in the genome. TR-1 cells also showed very little

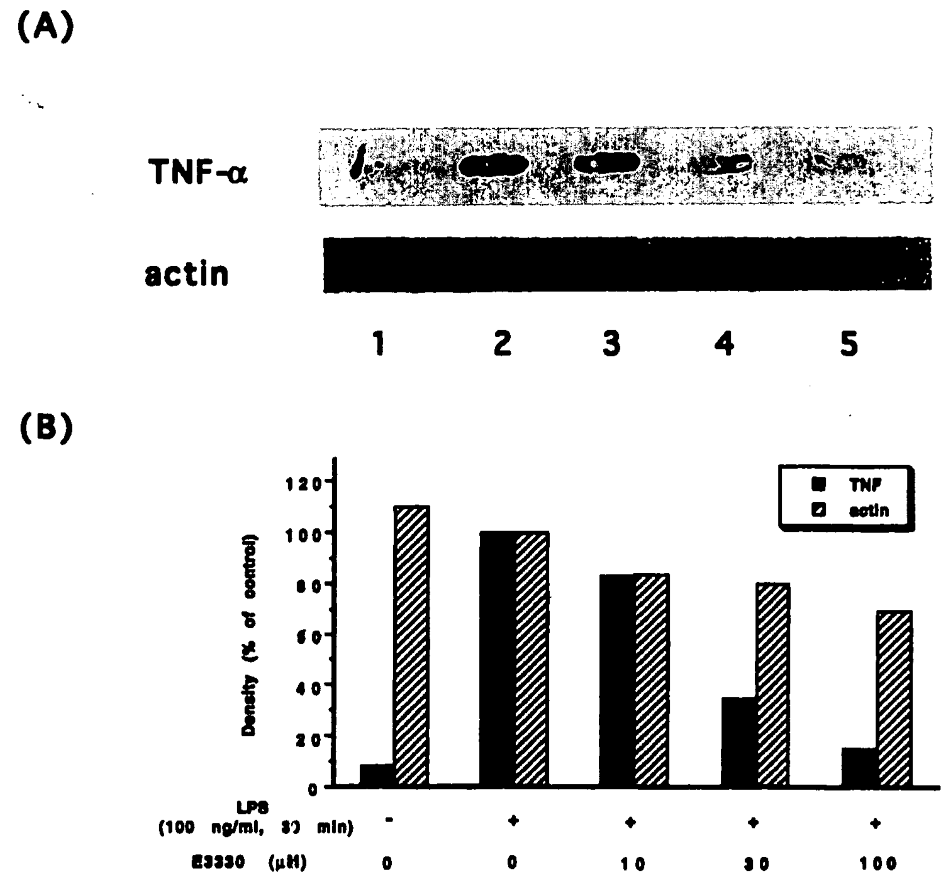

Fig. 4. Inhibitory effect of E3330 on steady state level of TNF-α mRNA. A, Nuclear run-on assay. Human monocytes were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS for 30 min. Isolation of nuclei and elongation, purification, and hybridization of RNA are described in Materials and Methods. *Lane 1*, without LPS; *lane 2*, LPS (100 ng/ml); *lane 3*, LPS (100 ng/ml) + E3330 (10 μM); *lane 4*, LPS (100 ng/ml) + E3330 (30 μM); *lane 5*, LPS (100 ng/ml) + E3330 (100 μM). B, Densitometry of the spots. The value of *lane 2* was defined as 100%. The 100% values of TNF-α and actin were 9,345 and 11,160, respectively. *Solid bars*, TNF-α mRNA; *hatched bars*, actin mRNA.

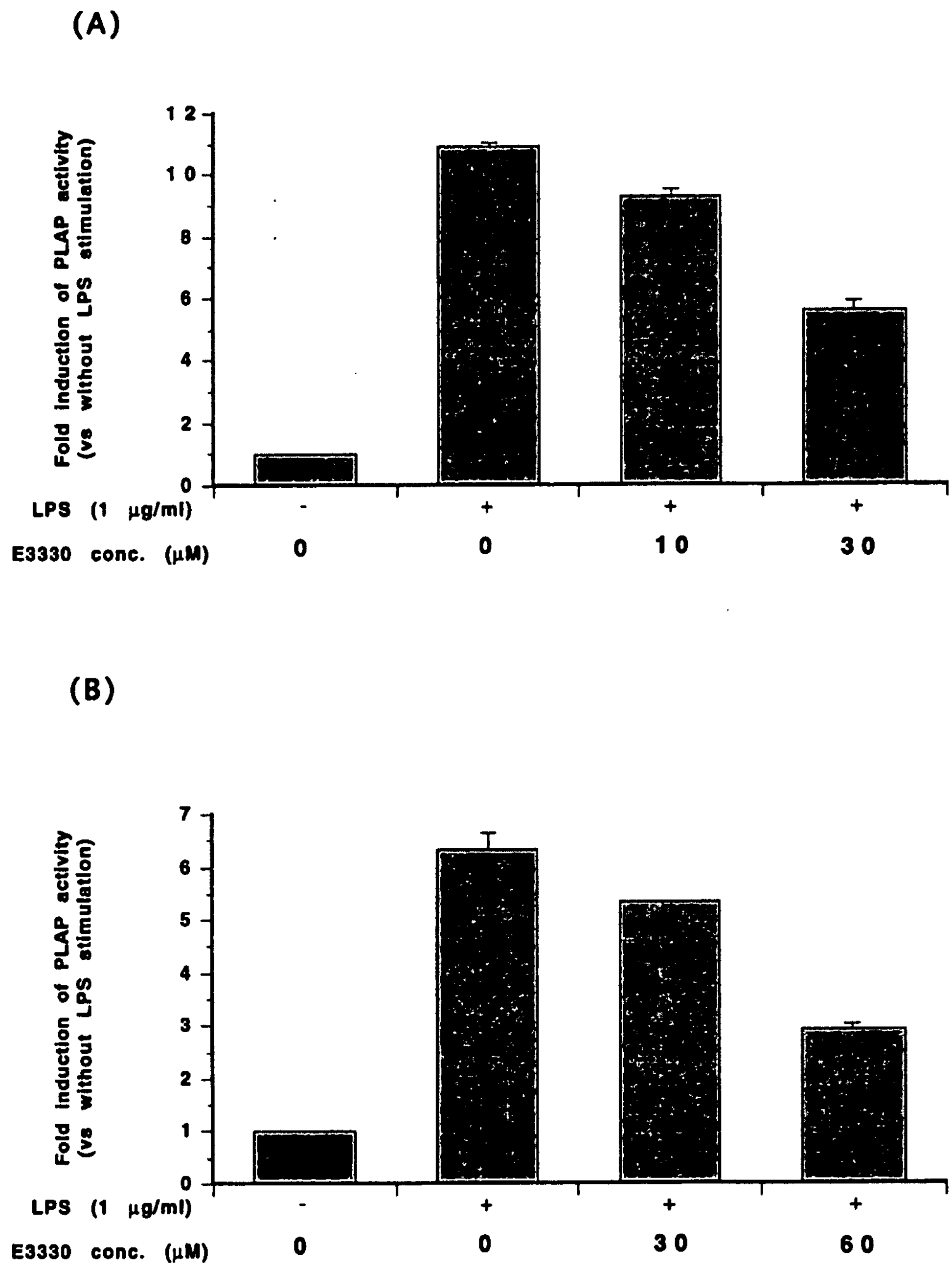

Fig. 5. Inhibitory effect of E3330 on transcription of TNF-α gene induced by LPS. A, Effect of E3330 on PLAP transcription of LPS-stimulated Raw264.7 cells. TNF-α PLAP plasmid was transiently transfected into the Raw264.7 cells with the DEAE-dextran method (see Materials and Methods). After the transfection, the cells were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS (1 μg/ml) for 48 hr. Culture supernatant was collected from each well and assayed for alkaline phosphatase activity. The amounts of alkaline phosphatase activity were estimated as arbitrary units by chemiluminescence counting with a microLumat LB 96. The linearity of the standard placental alkaline phosphatase activity was maintained from 0.25 ng/ml (256 $\pm$ 13) to 25 mg/ml (3,480,546 $\pm$ 40,641, mean $\pm$ standard error; five experiments). The mean value of 1.0-fold induction was 23,943 $\pm$ 467 (three experiments). Each value is the mean $\pm$ standard error of three experiments. B, Effect of E3330 on PLAP transcription of LPS-stimulated TR-1 cells. TR-1 cells were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS (100 ng/ml) for 18 hr. The mean value of 1.0-fold induction was 415 $\pm$ 6. Each value is the mean $\pm$ standard error of three experiments.

PLAP activity before stimulation, whereas stimulation of the cells with LPS led to a ~6.8-fold increase in PLAP activity. As shown in Fig. 5B, E3330 inhibited the production of PLAP in a dose-dependent manner. The inhibitory effect of E3330 at 60 μM was 64% and LDH release from the E3330-treated cells was <10% compared with that of lysed cells, indicating that the inhibitory effect of E3330 is not due to cytotoxicity. These results indicate that E3330 inhibits the induced expression of TNF-α-PLAP reporter gene.

NF-κB is related to transcription of TNF-α gene. NF-κB is a critical transcriptional factor of several genes involved in immune and inflammatory responses (13, 14). It has been reported that transcription of murine TNF-α gene was regulated by NF-κB activation (11, 12). We identified four NF-κB recognition site-like sequences located at −634 (κB-1), −605 (κB-2), −220 (κB-3), and −105 (κB-4) bp from the transcription start site in the 5′ upstream region of human TNF-α gene. The locations of these NF-κB recognition site-like sequences are indicated in Fig. 6A. To characterize the functional role of the putative NF-κB binding sites, we introduced mutations by using synthetic oligonucleotides (Fig. 6B), and we ligated them with PLAP reporter plasmid to generate TNF-α-PLAP containing mutant NF-κB recognition site-like sequences. These constructs were transformed into Raw264.7 cells, and their

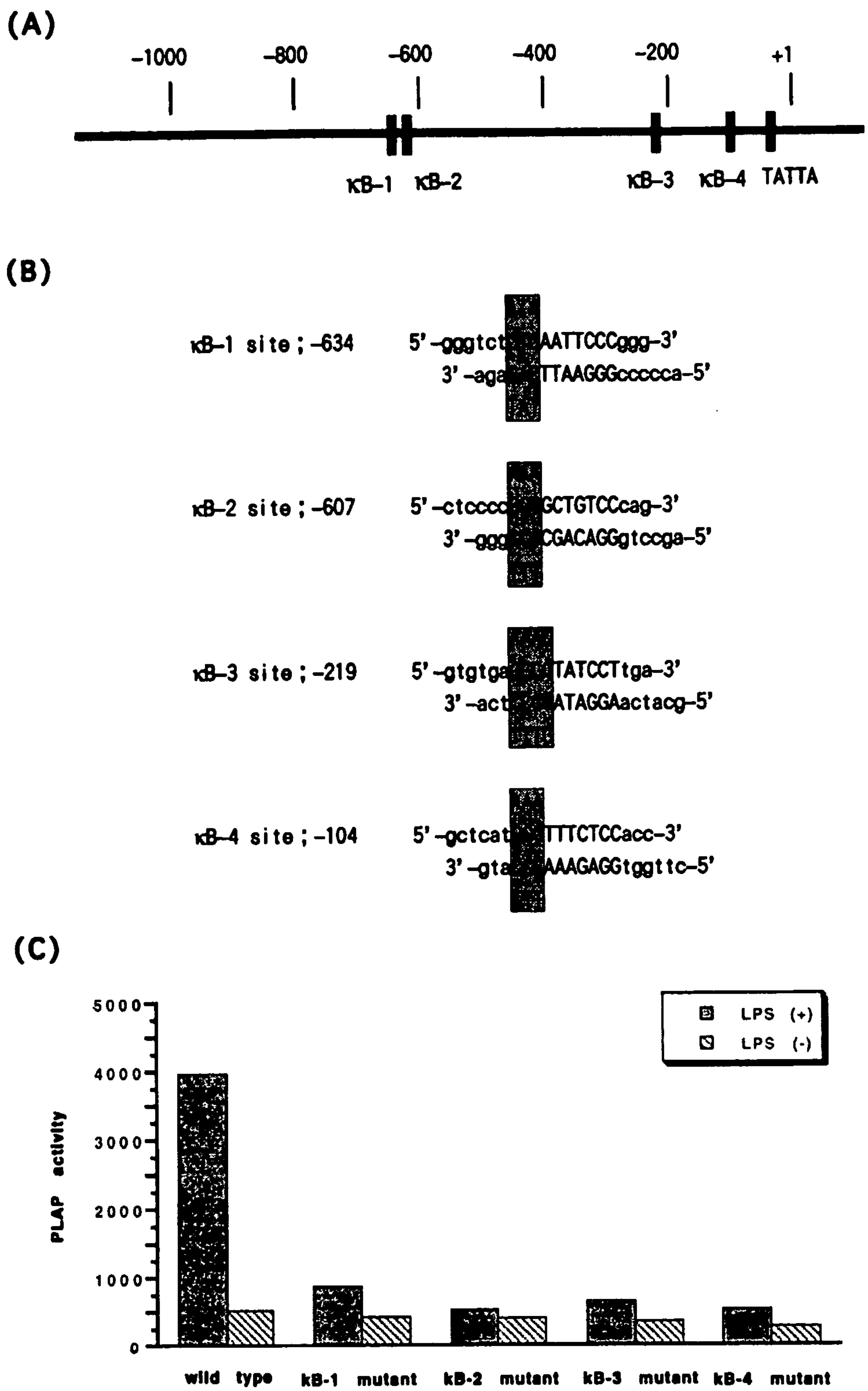

Fig. 6. Effects of NF-κB binding site mutations in the TNF-α 5′ upstream region on transcription. A, Structure of the human TNF-α promoter. The sequence of the NF-κB-like sites and their locations relative to the mRNA start site are indicated. B, Mutant sequences of NF-κB sites in the TNF-α 5′ upstream region. *Shaded regions*, nucleotide changes in the mutants. C, Effects of NF-κB binding site mutations in the TNF-α 5′ upstream region on transcription. Plasmids containing NF-κB binding site-mutated forms of the TNF-α 5′ upstream region were transfected into Raw264.7 cells with the DEAE-dextran method. The cells were stimulated with LPS (1 μg/ml) for 18 hr, and the amounts of alkaline phosphatase activity in the culture supernatant were estimated. *Solid bars*, LPS-stimulated cells; *hatched bars*, unstimulated cells.

inducibility in response to LPS stimulation was examined. We chose Raw264.7 cells because this cell line showed excellent transfection competency, was easy to transfect, and was suitable for comparing the transcription levels among plural plasmids. As shown in Fig. 6C, the expression of wild-type TNF-α-PLAP reporter gene was low before induction, whereas this gene was highly inducible in Raw264.7 cells on stimulation with LPS. On the other hand, induced expression of the mutant genes was dramatically reduced. These data indicated that each of the NF-κB recognition site-like sequences plays a critical role in LPS stimulation of human TNF-α gene transcription.

Induction and characterization of NF-κB in nuclei of LPS-stimulated human monocytes and inhibitory effect of E3330 on induction of NF-κB activity detected by binding to oligonucleotides corresponding to TNF-α NF-κB-recognition site-like sequences. NF-κB p50/p65 heterodimer is present in the cytosol of resting cells (13, 14). After stimulation of the cells with various agents, the cytosolic NF-κB/IκB complex is dissociated, and free NF-κB translocates to the nuclei. We performed gel retardation assay with four oligonucleotides having NF-κB recognition site-like sequences in the TNF-α 5′ upstream region and the same nuclear extract prepared from LPS-stimulated human monocytes (Fig. 7A). Although a small amount of a faster-migrating band was detected in the nuclei of resting monocytes, the slower-migrating band in the nuclei increased after stimulation of the cells with LPS (10 ng/ml) for 60 min. The result with a ^{32}P-labeled κB-1 oligonucleotide showed that the amount of faster-migrating band in the nuclei was unchanged, whereas the increase in the slower-migrating band after stimulation for 60 min amounted to 2.4-fold from the resting level on the basis of densitometry with a BAS 2000 image analyzer. The intensity of binding was in the order of κB-1 > κB-2 > κB-4 > κB-3. There was no difference in migration among the κB oligonucleotides. These results indicate that activated-NF-κB binds to all four κB sites of TNF-α gene.

To confirm the specificity of these bands that were detected with gel shift assay, we carried out competition assay with cold oligonucleotides. These bands between ^{32}P-labeled κB-1 oligonucleotide and nuclear extracts from LPS-stimulated human monocytes were effectively eliminated by competition by excess unlabeled κB-1 oligonucleotide but not by an unrelated oligonucleotide of cAMP-responsive element (-TGACGTTCA-) (Fig. 7B, *left*). Furthermore, the results of supershift assay with specific antibodies reactive with NF-κB protein suggested that these bands were NF-κB proteins. As shown in Fig. 7B (*right*), the faster- and slower-migrating bands were both shifted by antibody reactive with p50, whereas only the latter band was shifted by antibody reactive with p65. Anti-p52, anti-Rel, and anti-relB antibodies did not affect the migration of these bands (data not shown). On the other hand, the band between ^{32}P-labeled κB-1 oligonucleotide and nuclear extracts from unstimulated human monocytes was shifted only by anti-p50 antibody (data not shown).

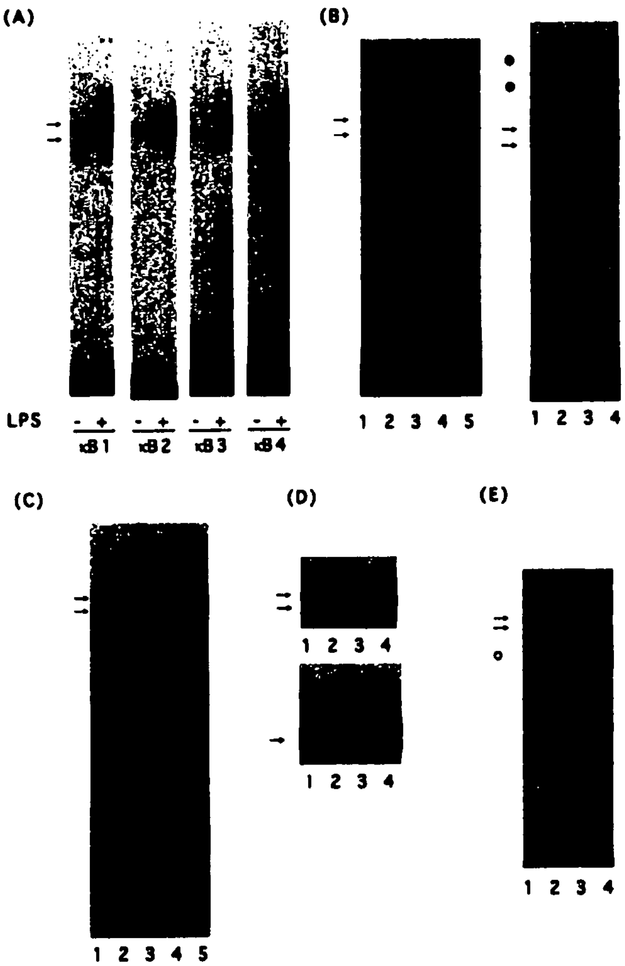

Fig. 7. Effect of E3330 on NF-κB activity. A, Detection of NF-κB binding by TNF-α NF-κB oligonucleotides. Nuclear extracts from LPS (10 ng/ml, 60 min)-stimulated human monocytes were incubated with ^{32}P-labeled TNF-α κB-1, κB-2, κB-3, and κB-4 double-stranded oligonucleotide. *Arrows*, specific binding. B, Specificity of bindings detected by TNF-α NF-κB-1 oligonucleotides. *Left*, competition for binding of LPS-induced nuclear NF-κB with NF-κB-related or unrelated oligonucleotides. Nuclear extracts from human monocytes stimulated with LPS (10 ng/ml) for 60 min were incubated with ^{32}P-labeled double-stranded TNF-α κB-1 oligonucleotide in the presence or absence of NF-κB-related or unrelated oligonucleotides. *Lane 1*, without competitor; *lane 2*, 10-fold excess of unlabeled double-stranded TNF-α κB-1 oligonucleotide; *lane 3*, 100-fold excess of unlabeled double-stranded TNF-α κB-1 oligonucleotide; *lane 4*, 10-fold excess of unlabeled double-stranded cAMP-responsive element oligonucleotide; *lane 5*, 100-fold excess of unlabeled double-stranded cAMP-responsive element oligonucleotide. *Arrows*, specific binding. *Right*, identification of NF-κB in the gel shift assay. Nuclear extracts from human monocytes stimulated with LPS (10 ng/ml) for 60 min were incubated with ^{32}P-labeled double-stranded TNF-α κB-1 oligonucleotide, followed by the addition of antibodies at 4° for 60 min. *Lane 1*, without antibody; *lane 2*, anti-p50 antibody; *lane 3*, anti-p65 antibody; *lane 4*, anti-p52 antibody. *Arrows*, specific binding. ●, Supershifted binding. C, Inhibitory effect of E3330 on the induction of NF-κB activity in human monocyte nuclei detected by binding to double-stranded TNF-α κB oligonucleotides. Human monocytes were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS for 120 min. The nuclear extracts prepared from these cells were incubated with ^{32}P-labeled double-stranded oligonucleotide of TNF-α κB-1. *Lane 1*, without LPS; *lane 2*, LPS (10 ng/ml); *lane 3*, LPS (10 ng/ml) + E3330 (10 μM); *lane 4*, LPS (10 ng/ml) + E3330 (30 μM); *lane 5*, LPS (10 ng/ml) + E3330 (100 μM). *Arrows*, specific binding. D, No inhibitory effect of E3330 on the direct binding of NF-κB to TNF-α κB-1 oligonucleotide. *Top*, effect of E3330 on the direct binding of nuclear extracts from LPS-stimulated human monocytes to TNF-α κB-1 oligonucleotide. Nuclear extracts from human monocytes stimulated with LPS (10 ng/ml) for 60 min were incubated with ^{32}P-labeled double-stranded TNF-α κB-1 oligonucleotide in the presence or absence of E3330. *Lane 1*, without drug; *lane 2*, E3330 (10 μM); *lane 3*, E3330 (30 μM); *lane 4*, E3330 (100 μM). *Arrows*, specific binding. *Bottom*, effect of E3330 on the direct binding of human recombinant p50 protein to TNF-α κB-1 oligonucleotide. Human recombinant p50 protein was incubated with ^{32}P-labeled double-stranded TNF-α κB-1 oligonucleotide in the presence or absence of E3330. The order of lanes was the same as on the *top*. *Arrow*, p50 homodimer binding. E, Effect of E3330 on the induction of NF-κB binding activity to HIV-1 κB oligonucleotide in nuclei of human monocytes. The experimental procedure was the same as in C except for the use of HIV-1 κB oligonucleotide instead of TNF-α κB-1 oligonucleotide. *Lane 1*, without LPS; *lane 2*, LPS (10 ng/ml); *lane 3*, LPS (10 ng/ml) + E3330 (10 μM); *lane 4*, LPS (10 ng/ml) + E3330 (30 μM). *Arrows*, specific binding. ○, Nonspecific binding.

Similar results were obtained by using κB-2, κB-3, and κB-4 oligonucleotides instead of κB-1 oligonucleotide with nuclear extract of LPS-stimulated human monocytes (data not shown). These data indicated that the faster-migrating band was composed of p50 homodimer and the slower-migrating band was composed of p50/p65 heterodimer, implying that stimulation with LPS induces p50/p65 heterodimer in nuclei of human monocytes. Next, we examined the effect of E3330 on the appearance of NF-κB binding activity in the nuclei. The results of gel shift assay with a ^{32}P-labeled κB-1 oligonucleotide and nuclear extracts of LPS-stimulated human monocytes are shown in Fig. 7C. E3330 inhibited the induction of slower-migrating NF-κB binding to κB-1 oligonucleotide in a dose-dependent manner. The inhibitory effect of E3330 at 30 μM was 92%. Similar results were obtained by using κB-2, κB-3, and κB-4 oligonucleotides instead of κB-1 oligonucleotide (data not shown).

The effect of E3330 on direct binding of NF-κB to κB-1 oligonucleotide is shown in Fig. 7D. This compound did not inhibit the direct binding of nuclear extracts from LPS-stimulated monocytes (*top*) or human recombinant p50 homodimer (*bottom*) to κB-1 oligonucleotide, even at 100 μM. These results suggest that E3330 inhibits NF-κB activation and/or translocation to the nuclei from cytosol.

Although NF-κB-recognized sequences have been found the in 5′ upstream regions of many genes, HIV-1 is of particular interest because HIV-1 is the causal retrovirus of AIDS (31). It has been reported that tandem NF-κB sequences exist in the LTR of HIV-1, and NF-κB positively regulates transcription of this gene (3, 13, 14, 28). Next, we examined the inhibitory effects of E3330 on the induction of NF-κB binding activity through the use of gel shift assay with HIV-1 κB oligonucleotide (Fig. 7E). E3330 inhibited the induction of the NF-κB binding activity of the slower-migrating band. The inhibitory effect at 30 μM amounted to 86%.

Inhibitory effect of E3330 on expression of NF-κB reporter gene. TNF-α gene activation in response to LPS stimulation requires four putative NF-κB binding sites in the 5′ upstream region of the TNF-α gene. To determine the effect of E3330 on NF-κB activation, Raw264.7 cells were transfected with other NF-κB activatable plasmids, and the effect of E3330 on the gene transcription was measured (Table 1). When Raw264.7 cells that had been transiently transfected with HIV-1 κB-PLAP, containing four copies of the HIV-1 κB motif, were stimulated with LPS (1 μg/ml) for 48 hr, PLAP activity in the culture supernatant increased to 3.2 times the basal level. E3330 at 30 μM reduced the induction of PLAP expression to 81% of the control. Transfection with HIV-1 LTR-PLAP and stimulation with LPS under the same conditions gave a 2.6-fold induction of PLAP enzyme. E3330 at 30 μM reduced the induction of PLAP activity to 41% of the control. In addition, when Raw264.7 cells were transfected with pSV2 PLAP and stimulated with LPS under the same conditions, PLAP activity increased to 12.0 times the basal activity, and E3330 at 30 μM decreased the induction of PLAP activity to 71% of the control. During these experiments, we did not detect significant cytotoxicity with the MTT assay, so E3330 clearly affected the NF-κB-containing promoters, but the degree of the inhibition differed, depending on the reporter constructs. Possibly, the active NF-κB concentration required in the nuclei for gene activation might be different for each NF-κB binding element or promoter. In addition, other inducible transcription factors might be involved in the activation of pSV2 and HIV-1 LTR and act synergistically in the promoter activation. These results suggest that E3330 can inhibit NF-κB activation or a signaling pathway that leads to NF-κB activation.

TABLE 1

Inhibitory effect of E3330 on transcriptional activity of HIV-1 κB- and HIV-1 LTR-reporter gene

The plasmids were transfected into Raw 264.7 cells according to the DEAE-dextran method. The cells were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS (1 μg/ml) for 48 hr. Culture supernatant was collected from each well and assayed for alkaline phosphatase activity.

Plasmid	LPS	E3330	PLAP activity[a]	Percent of induced PLAP activity
	μg/ml	*μM*		*%*
HIV-1 κB-PLAP	0	0	0.1 ± 0.1	0
	1	0	2.6 ± 0.1	100
	1	30	1.3 ± 0.1	17
HIV-1 LTR-PLAP	0	0	1.0 ± 0.1	0
	1	0	3.2 ± 0.0	100
	1	30	1.9 ± 0.1	40
pSV2-PLAP	0	0	1.0 ± 0.0	0
	1	0	12.1 ± 0.1	100
	1	30	8.9 ± 0.2	71

[a] The amounts of alkaline phosphatase activity were estimated as arbitrary units by chemiluminescence counting with an LB96P. The mean values of 1.0-fold induction were 40,996 ± 4,251 (HIV-1 κB-PLAP), 54,096 ± 2,917 (HIV-1 LTR-PLAP), and 13,321 ± 210 (pSV2-PLAP) for three experiments.

Inhibitory effect of E3330 on IκB-α degradation. NF-κB is present in the cytosol of resting cells as a complex with an inhibitory protein, IκB-α (13, 14). A variety of cellular stimuli cause the dissociation of NF-κB from IκB-α, allowing free NF-κB to translocate to the nuclei and activate gene expression. The translocation of NF-κB is preceded by the phosphorylation and degradation of IκB-α (33–35). We therefore examined the effect of E3330 on IκB-α phosphorylation and degradation. The results of Western blotting with anti-IκB-α antibody showed that IκB-α was separated with SDS-polyacrylamide gel electrophoresis as a 37-kDa band from resting human monocytes (Fig. 8, *lane 1*). After the stimulation of human monocytes with LPS (100 ng/ml) for 30 min, the band of IκB-α decreased, and the phosphorylated form of IκB-α appeared as a slower-moving band (Fig. 8, *lane 2*). E3330 had no distinct effect on the appearance of slower-moving band, but it inhibited the decay of native IκB-α in a dose-dependent manner (Fig. 8, *lanes 3–5*). These results indicated that E3330 might inhibit some step in the mechanisms, leading to the degradation of IκB-α protein.

Effect of E3330 on superoxide anion production. Because active oxygen species serve as common messengers that activate NF-κB (13, 14, 32, 36, 37), we examined the effect of E3330 on superoxide anion production of human monocytes (Table 2). When human monocytes attached to a plastic plate were stimulated with LPS (1 μg/ml), PMA (10 nM), or fMLP (10 μM), superoxide production was 11.1 ± 0.6, 12.3 ± 1.3, or 22.1 ± 1.7 nmol/ml/60 min, respectively. The inhibitory effect of E3330 at 100 μM amounted to 74% for LPS, 81% for PMA, and 84% for fMLP. In the superoxide production system with xanthine

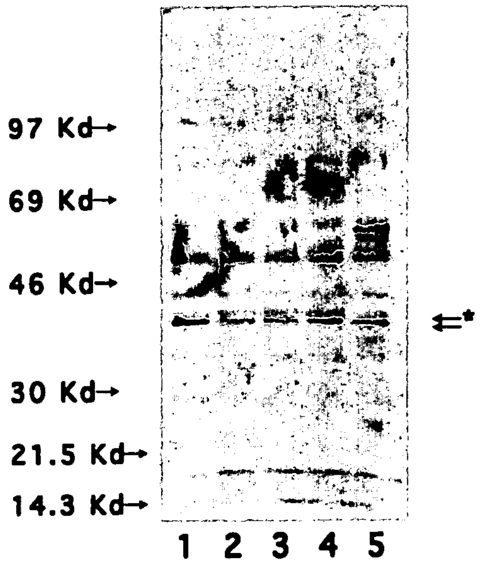

Fig. 8. Inhibitory effect of E3330 on phosphorylation of IκB-α. Human monocytes were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS for 30 min. Preparation of the whole-cell lysate and electrophoresis and blotting were as described in Materials and Methods. The membrane was analyzed for the degradation of IκB-α proteins with rabbit anti-IκB-α antibody and horseradish peroxidase-linked donkey anti-rabbit IgG antiserum. The membranes were dried, incubated in ECL reagents, covered with Saran wrap, and exposed to ECL hyperfilms. *Lane 1*, without LPS; *lane 2*, LPS (10 ng/ml); *lane 3*, LPS (10 ng/ml) + E3330 (10 μM); *lane 4*, LPS (10 ng/ml) + E3330 (30 μM); *lane 5*, LPS (10 ng/ml) + E3330 (100 μM). *Arrows*, IκB-α proteins. *, Phosphorylated form of IκB-α.

oxidase and xanthine, the corresponding value was only 22%. These results suggest that one mechanism of E3330 action on NF-κB activation might be inhibition of superoxide anion production.

Discussion

E3330 is a novel synthetic quinone derivative that was discovered in our laboratories and has protective effects against endotoxin-mediated hepatitis in mice and galactosamine-induced hepatitis in rats (8, 9). The main mechanism of the protective effect of E3330 in the animal models is considered to be inhibition of TNF-α generation. E3330 inhibits TNF-α generation from several types of macrophages/monocytes, and the effect is considered to be based on the suppression of TNF-α mRNA expression (7). The results of the nuclear run-on assay indicated that E3330 decreases the transcription rate of TNF-α mRNA without altering that of actin mRNA (Fig. 4). Furthermore, E3330 decreased LPS-induced PLAP gene expression in Raw264.7 cells transiently transfected with TNF-α reporter gene and in TR-1 cells in which TNF-α reporter gene was stably integrated in the genome (Fig. 5, A and B). These data strongly suggest that the inhibitory effect of E3330 on TNF-α biosynthesis is caused at least in part by the suppression of transcriptional activation.

There have been many studies on critical regulatory regions for human TNF-α gene transcription. Deletion and/or mutation experiments showed that significant regions include the region −95 to −35 upstream from the transcription start site for PMA-stimulated U937 cells (10), the region −125 to −82 upstream for TNF-stimulated K652 cells (38), the region −100 to −74 upstream for PMA-treated Jurkat cells expressing C/EBP-β (39), and the region −115 to −98 upstream for PMA-induced Hut78 cells (40). On the other hand, it has been reported that NF-κB binding sites (especially that −510 to −501 upstream from the transcription start site) are important for LPS-stimulated TNF-α transcription in murine peritoneal macrophages (11, 12). NF-κB is a critical regulator of several genes involved in immune and inflammatory responses (13, 14). We therefore examined the role of NF-κB recognition sites in the 5′ upstream region of the human TNF-α gene.

Because the NF-κB binding sequence at −510 to −501 upstream exists in murine TNF-α gene but not in human TNF-α gene, transcription of the gene is presumably regulated by other NF-κB binding sequences. We searched for NF-κB recognition site-like sequences in the 5′ upstream region of human TNF-α gene and found sites located at −634 (κB-1), −605 (κB-2), −220 (κB-3), and −105 (κB-4) bp from the transcription start site (Fig. 6A). We prepared human TNF-α PLAP reporter plasmids, each containing one NF-κB site mutation in the 1.4-kb 5′ upstream region, and transiently transfected Raw264.7 cells with these plasmids. We chose Raw264.7 cells because this cell line showed excellent transfection competency and was suitable for comparison of the transcription level among plural plasmids. Although Raw264.7 cells are murine cell lines, the cells that were transfected with wild-type human TNF-α-reporter plasmids showed high inducibility (Fig. 6C). Furthermore, we confirmed the presence of NF-κB in nuclear extracts from LPS-stimulated Raw264.7 through binding studies to oligonucleotides corresponding to the human TNF-α NF-κB-recognition site-like sequences described above (data not shown). The suppression of transcriptional activity in Raw264.7 cells that were transfected with various human TNF-α-reporter plasmids containing a single NF-κB site mutation suggested that each of the NF-κB sites plays a critical role in LPS-stimulated TNF-α transcription.

Through the use of gel shift assay with oligonucleotides corresponding to human TNF-α NF-κB-recognition site-like sequences, two specific bands of NF-κB was clearly detected in the nuclear extracts prepared from LPS-stimulated human monocytes (Fig. 7A). The results of supershift assay indicated that the faster-migrating band was composed of p50 homodimer and the slower-migrating band was composed p50/p65 heterodimer (Fig. 7B). The amount of p50/65 heterodimer increased after stimulation with LPS from 30 to 120 min, but that of p50 homodimer did not. These data suggest that the increase of p50/65 heterodimer in the nuclei may lead to up-regulation of TNF-α transcription. This result should be confirmed by transcription experiments involving cotransfection with p50 and p65 protein expression vectors in the TR-1 and Raw264.7 cell systems. The order of binding activity between NF-κB (p50/65 heterodimer) and κB oligonucleotides was κB-1 > κB-2 > κB-4 > κB-3. Recently, it has been reported that the 5′ upstream region of the human TNF-α gene contains four potential NF-κB recognition sites at positions −104, −220, −605, and −633, and the order of binding strength is −605 > −104 > −220 > −633 (41). The

TABLE 2
Inhibitory effects of E3330 on the production of superoxide anion by human monocytes

	Stimuli	E3330 (μM)	O_2^- production	Percent of control
			nmol/ml/60 min	%
Human monocytes[a]	LPS (1 μg/ml)	0	11.0 ± 0.6	100
		10	8.8 ± 1.1	79
		30	7.0 ± 0.5	64
		100	2.9 ± 1.0	26
	PMA (10 nM)	0	22.1 ± 1.7	100
		10	14.5 ± 1.7	66
		30	14.0 ± 0.6	63
		100	4.2 ± 0.1	19
	fMLP (10 μM)	0	12.3 ± 1.3	100
		10	9.8 ± 0.2	80
		30	6.9 ± 0.2	56
		100	2.0 ± 0.8	16
Xanthine oxidase/xanthine[b]	Xanthine oxidase (10 m units/ml) Xanthine (0.1 mM)	0	14.9 ± 0.1	100
		10	14.8 ± 0.3	99
		30	13.5 ± 0.1	91
		100	11.6 ± 0.1	78

[a] Human monocytes were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS (1 μg/ml), PMA (10 nM), or fMLP (10 μM) for 60 min. Each value is the mean ± standard error of three experiments.
[b] Superoxide anion was generated with a xanthine oxidase/xanthine system in the presence or absence of E3330 for 30 min. Each value is the mean ± standard error of three experiments.

difference of that order from ours may be due to differences of experimental conditions, including the cells (e.g., freshly isolated human monocytes versus Mono Mac 6 cells), the culture conditions (e.g., autologous heat-inactivated serum versus fetal calf serum), and so on.

Next, we investigated the effect of E3330 on the appearance of NF-κB in the nuclei of LPS-stimulated human monocytes. Recently, it was reported that cyclosporin A inhibits TNF-α generation by anti-CD3-, anti-T cell receptor-α/β-, or ionomycin-stimulated murine Ar 5 T cells due to inhibition of the induction of NF-κB binding to the site located at −104 bp from the transcription start site (42). Our results suggest that the inhibitory effect of E3330 on TNF-α mRNA biosynthesis is caused by inhibition of the induction of NF-κB binding to the four κB sites of the TNF-α 5′ upstream region (Fig. 7C). Because E3330 did not inhibit the direct binding of nuclear extracts from LPS-stimulated monocytes and of human recombinant p50 homodimers to κB-1 oligonucleotide (Fig. 7D), the compound might affect signal transduction, leading to NF-κB activation after LPS stimulation.

NF-κB is known to activate not only TNF-α gene but also many other genes, including HIV-1 (13, 14). Tandem NF-κB-recognized sequences exist in the LTR of HIV-1 (28). It has been reported that monocytes/macrophages infected with HIV-1 play an important role as a reservoir of HIV-1, and LPS is a potent stimulator of HIV-1 expression in the cells (43, 44). We examined whether the inhibitory effect of E3330 on the induction of NF-κB binding activity is recognized by an oligonucleotide corresponding to HIV-1 κB. E3330 inhibited the induction of NF-κB recognized by HIV-1 κB oligonucleotide in the same way as seen with TNF-α κB oligonucleotides (Fig. 7E). The results of the HIV-1 κB plasmid transfection experiment suggest that the inhibitory effect of E3330 on the transcriptional activity may be caused by inhibition of NF-κB activation (Table 1). Similar results were obtained in Raw264.7 cells transfected with HIV-1 LTR-PLAP and TNF-α PLAP that contain NF-κB binding sites (Table 1 and Fig. 5A). These results indicate that the inhibitory effect of E3330 on the transcription might be specific to NF-κB-inducible genes.

NF-κB activation is regulated by its cytoplasmic inhibitor IκB-α (13, 14). The activation of NF-κB is associated with phosphorylation and degradation of IκB-α (32, 34, 35, 45, 46). The inducible phosphorylation of IκB-α is not sufficient for its dissociation from NF-κB (45–47) but is a signal for its proteolytic degradation by ubiquitin-dependent proteasome (34, 45, 48). This degradation process is absolutely required for NF-κB activation. To clarify the effect of E3330 on the IκB-α degradation, we performed Western blotting analysis with anti-IκB-α (Fig. 8). IκB-α was detected at 37 kDa through SDS-polyacrylamide gel electrophoresis in resting human monocytes. After LPS stimulation, the phosphorylated form of IκB-α was detected at a position corresponding to larger molecular size. Native IκB-α decreased as a result of the phosphorylation and the subsequent degradation (Fig. 8, *lane 2*). E3330 inhibited the decay of native IκB-α protein (Fig. 8, *lanes 4 and 5*). These data suggest that the inhibitory effect of E3330 on the induction of NF-κB is due to repression of IκB-α degradation.

Recently, it was reported that oxygen radicals are involved in the activation of NF-κB (13, 14, 36, 37). Therefore, we examined the effect of E3330 on superoxide anion production by human monocytes. The amounts of superoxide anion production by plastic plate-attached monocytes stimulated with LPS were comparable with those after PMA or fMLP stimulation. E3330 suppressed the production of superoxide anion in monocytes stimulated with not only LPS but also PMA and fMLP, although this compound showed no scavenging activity (Table 2).

The mechanism by which the inhibitory effect of E3330 on oxygen radical production would lead to suppression of IκB-α degradation is unclear. One possibility is that oxygen radicals might regulate the phosphorylation level of IκB-α. This is supported by a recent study demonstrating that the phosphorylation of IκB-α is blocked by the antioxidant pyrrolidine dithiocarbamate (32). The degradation of IκB-α is preceded by phosphorylation on Ser^{32} and Ser^{36}

(33–35). If phosphorylation of IκB-α is essential for IκB-α degradation, the inhibitory effect of E3330 on the decay of native IκB-α protein might reflect modulation by E3330 of the phosphorylation or dephosphorylation of IκB-α, although the compound did not distinctly suppress the formation of phosphorylated IκB-α (Fig. 8, *lanes 4 and 5*). It has been reported that protein kinase C and some tyrosine kinases can phosphorylate IκB-α in cell-free systems (13, 14), but E3330 did not inhibit either protein kinase C or the tyrosine phosphorylation of $p56^{lyn}$, which is associated with CD14 in LPS-stimulated human monocytes (data not shown). These data suggest that E3330 might affect phosphorylation at some other step of signal transduction that is regulated by oxygen radicals after LPS stimulation. A second possibility is that IκB-α protein could be damaged directly by oxygen radicals. Oxidative damage is involved in the irreversible inactivation of α1-proteinase inhibitor by radicals and in the reversible regulation of glyceraldehyde-3-phosphate dehydrogenase by oxidant stress (36).

In summary, we clarified that one of the inhibitory mechanisms of E3330 on TNF-α generation is the inhibition of NF-κB activation through suppression of IκB-α degradation. The inhibitory effect of E3330 on IκB-α protein degradation might be a consequence of a decrease of superoxide anion production.

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